AFLATOXIN ELIMINATION WORKSHOP

Atlanta, Georgia

October 23-24, 1995

Draft

AFLATOXIN ELIMINATION WORKSHOP
Atlanta, Georgia
October 23-24, 1995

Aflatoxin is recognized as a serious food safety hazard by most countries of the world. Producing food free of aflatoxin today requires a truly national effort and, particularly, the cooperation of both government and industry. The Agricultural Research Service (ARS) and the commodity groups representing peanuts, corn, cottonseed, and tree nuts recognize the importance of a strong national research effort to eliminate aflatoxin as a food safety threat.

This Aflatoxin Elimination Workshop, held in St. Louis, Missouri, is the seventh such yearly meeting held to review the ARS supported aflatoxin research and provide a forum for interested scientists to come together to discuss common problems and their potential solutions among themselves and with members of the industry. Thus, although many of these scientists are performing very fundamental studies researchers and representatives of industries affected by aflatoxin who attend the workshop gain a very clear idea of where their research is leading and the impact it will have on society. Also, this workshop provides the opportunity for gains in cost effectiveness of research by the recognition of common approaches and by sharing relevant information across commodities. This workshop has come to be recognized as the premier national meeting for advances leading to methods to eliminate aflatoxin.

Recent advances that were not even thought possible two or three years ago have been made in all segments of research to eliminate aflatoxin as a production and processing problem for peanuts, corn, cottonseed and tree nuts.

In the past the lack of gene encoded products which were clearly shown to directly inhibit A. flavus has caused great concern among the various laboratories engaged in genetic engineering. Scientists want to work only with genes that have the highest potential for limiting fungal growth and/or aflatoxin contamination since considerable effort goes into production of crops stably transformed with these foreign genes. However, confidence is building within the crop genetic engineering group that viable anti-A. flavus genes do indeed exist.

This is possible because we have developed techniques for screening germplasm both in the laboratory and in the field that were not available several years ago. For instance, previously scientists generated strains of A. flavus containing a reporter gene (GUS) fused to the ver-1 promotor (a gene involved in aflatoxin biosynthesis) and, using a simple fluorometric assay, showed that expression of GUS protein activity followed the same time course as aflatoxin synthesis. Now these same scientists have demonstrated the utility of these strains in identifying plant compounds which induce or inhibit aflatoxin synthesis at the transcriptional level.

In corn, the resistance identified is of more than one type, that is, it may be associated with waxes on the surface of the kernel, resistance to kernel splitting, or even associated with silk tissue, and thus may prove to be additive. However, we do not yet know how

many resistance mechanisms acting in concert will be required to yield useful resistance in commercial varieties. With corn, the consensus is that resistance from different sources with different genes (mechanisms) must first be crossed into elite inbreds and result in high yielding resistant inbreds. The resistance will then need to be incorporated into high yielding hybrids that will be accepted on the basis of yield and other characteristics in years that aflatoxin is not a potential problem.

A series of accomplishments in pistachio, fig and walnut, outlined on pages 57-58, provide information on the basic ecology of aflatoxigenic fungi in these crops and provide the basis for recommendations for growers and processors on how to reduce aflatoxin contamination.

The research to develop the use of atoxigenic strains of Aspergillus flavus to prevent aflatoxin contamination of cottonseed has progressed and has stimulated industry interest in large scale applications to the commercial crop in Arizona. With the assistance of the IR-4 Biopesticide Program, Peter Cotty at the Southern Regional Research Center has applied to the EPA for an Experimental Use Permit to allow treatment of limited commercial acreage of cotton in Arizona during the 1996 cotton season.

The recent successes in the multipronged strategies outlined in the 1995 Aflatoxin Elimination Workshop have dramatically increased optimism among ARS, University and Industry collaborators that methods to eliminate aflatoxin will be available for large scale implementation by the year 2000.

Most of the research is performed by the ARS, however an important addition to this core effort is provided through a competitive award program provided by Congressional appropriations. This program is a unique effort of the ARS and representatives of the peanut, corn, cotton, and tree nut industries. By extending the opportunity for the best university scientists to join the highly focused multithrust program, the rate of progress toward the elimination of aflatoxin is enhanced.

JANE F. ROBENS
Agricultural Research Service
Workshop Program Coordinator

November 22, 1995

TABLE OF CONTENTS

AFLATOXIN ELIMINATION WORKSHOP
ABSTRACTS OF PRESENTATIONS

AFLATOXIN ELIMINATION WORKSHOPS

New Orleans, LA	1988
Peoria, IL	1989
St. Louis, MO	1990
Atlanta, GA	1991
Fresno, CA	1992
Little Rock, AR	1993
St. Louis, MO	1994
Atlanta, GA	1995
Fresno, CA	1996

COOPERATING COMMODITY GROUPS

PEANUTS:	National Peanut Council
CORN:	National Corn Growers Association American Corn Millers Federation Corn Refiners Association
COTTONSEED:	National Cottonseed Products Association National Cotton Council
TREE NUTS:	Almond Board of California California Pistachio Commission DFA of California

CROP RESISTANCE - CONVENTIONAL BREEDING

Summary of Panel Discussion: Identification and Utilization of Aflatoxin Resistance in Germplasm

Panel Members: R. Brown, C. Holbrook, T. Gradziel, C. Martinson, T. Rocheford, D. White, D. Wicklow, and D. Wilson.

The panel was unanimous in their opinion that we have identified germplasm resistant to *Aspergillus flavus* and to aflatoxin production. Much of the progress is because we have the techniques for screening germplasm that were not available several years ago. Some of the mechanisms and inheritance of resistance are known, and others are being studied. It seems apparent that we are identifying both resistance to *Aspergillus flavus* per se and to aflatoxin production. A major concern is that we do not know what level of resistance is actually required for widely used commercial varieties.

The domestic and export almond markets presently demand zero to very low levels of aflatoxin contamination. To achieve such high levels of control, it may be necessary to integrate multiple genetic mechanisms for control of *Aspergillus* spp. with mechanisms for control of the Navel orangeworm which is important for initial fungal infection. Almond genotypes demonstrating suppressed fungal growth and/or suppressed toxin production in seed tissue have been identified, however, the response is variable with the environment. In addition, resistance to Navel orangeworm infestation through formation of a well-sealed, yet horticulturally acceptable, endocarp has been identified in a related wild specie and is being back-crossed into commercially acceptable almond. Presently, the cultivated variety "Mission" has the greatest antibiosis and nonpreference in seed and hull material for resistance to Navel orangeworm. It is expected that different mechanisms of resistance can be pyramided in order to create varieties with extremely high levels of resistance in most environments.

Research with peanuts also has identified different mechanisms and sources of resistance to *Aspergillus* spp. and aflatoxin production. To date, over 95% of the peanut core collection has been examined in a preliminary screen. Of the accessions which have been tested for at least three years, 16 exhibit low levels of aflatoxin contamination. In other studies, the link between low linolenic acid content and low aflatoxin production is being studied. Commercial varieties with low linolenic acid have been released and are in commercial production. If a significant link between low linolenic acid and aflatoxin exists, then resistant commercial varieties may already be available. In addition, studies of six peanut introductions with excessive root systems that are drought tolerant and have low aflatoxin contamination are being investigated. Unfortunately, many of the lines which have been identified as having lower aflatoxin contamination have less than acceptable agronomic characteristics. Resistant lines have been entered into a hybridization program to combine resistance to aflatoxin production with acceptable agronomic performance. Resistant lines are also being intermated to allow for combining of different genes for resistance to produce genotypes with even higher levels of resistance. It seems possible to combine the genes necessary for drought resistance, low linolenic acid content, and other mechanisms of resistance that have not been fully described into agronomically acceptable peanut cultivars.

In corn, several sources of resistance to Aspergillus ear rot and aflatoxin production have been identified by different researchers using completely different techniques. Resistance from one source may be partially due to waxes on the surface of the kernel. In other sources, resistance may be due to resistance to "kernel splitting". Resistance also has been identified that is associated with silk tissue. Corn mutants with a hexane soluble compound(s) that inhibit aflatoxin synthesis have been identified. With some sources of resistance, RFLP markers indicate that, in some cases, different chromosomal regions are responsible for Aspergillus ear rot resistance and for resistance to aflatoxin production. This is supported by work that suggests that a water soluble protein-like compound may be present in kernels that inhibits fungal growth and a different water soluble protein-like compound inhibits toxin production but not fungal growth. The consensus of opinion is that resistance from different sources with different genes (mechanisms) must be crossed into elite inbreds resulting in high yielding resistant inbreds and hybrids. The difficulty with utilizing resistance is that aflatoxin contamination of corn grain is not necessarily a limit to production in many areas of the United States every year. Therefore, resistance will need to be incorporated into high yielding hybrids that will be accepted on the basis of yield and other characteristics in years that aflatoxin is not a potential problem.

The panel was in complete agreement that significant progress has been made in identification of resistant germplasm. Research has provided both sources of resistance and techniques to utilize resistance for control of Aspergillus ear rot and aflatoxin production.

AFLATOXIN ELIMINATION WORKSHOP
Atlanta, Georgia, October 22-24, 1995

KERNEL 'SPLITTING' AND AFLATOXIN CONTAMINATION FOR CORN VARIETIES GROWN UNDER 'MILD' CLIMATIC CONDITIONS (LINCOLN, IL - 1994)

Donald T. Wicklow Phone (309)-681-6243
National Center for Agricultural Utilization Research,
ARS-USDA
1815 N. University Street
Peoria, IL 61604

In cooperative research with commercial corn seed producers we have found that B73 and OH43 inbreds often show breaks or tears in the seed coat over the germ, thus allowing A. flavus entry to the seed proper. In our field inoculation trials, evidence of this 'splitting' phenomenon is based upon a much higher frequency of BGYF kernels. Evidence is offered that some hybrids may become more susceptible to splitting as the "yield envelope is pushed" (Dr. Monte Miles, Mycogen). In an effort to measure the resistance of the living embryo to A. flavus infection and aflatoxin we modified the procedure of Brown et al., 1993. J. Food Protection 56: 967-971, to promote the expression of biochemical resistance. Grain samples (40 kernels) were first 'tempered' (98% RH; 15 C) for 5 days prior to needle wound-inoculation of the germ with A. flavus NRRL 3357. Inoculated kernels are then incubated for 5 days at 25 C (98% RH). Following incubation, the grain was examined for visible mold growth, A. flavus sporulation, BGYF, aflatoxin (Aflatest-Vicam, Inc.) and free ergosterol from the aflatoxin extract (HPLC). Ergosterol values enable us to estimate fungal biomass and extent of kernel infection. In a preliminary study, needle-inoculated grain sampled from 13 commercial hybrids gave aflatoxin values ranging from 62 ppb to 24,000 ppb, while aflatoxin values for 8 elite inbreds ranged from 2,200 ppb to 33,000 ppb. However, aflatoxin and ergosterol concentrations were highly sensitive to kernel moisture levels, precluding interpretations of varietal resistance. The level of free fungal ergosterol in the aflatoxin extract generally reflected the level of aflatoxin contamination. None of the corn varieties showed substantial fungal infection of the germ (i.e. sporulation, free ergosterol) with relatively low levels of aflatoxin. In 1995 we continued our cooperative work with Mycogen Plant Sciences, Lincoln, Illinois and Cargill Hybrid Seeds, Mt. Vernon, Indiana.

*This research is included in ARS Project 3620-42000-015-00D "Integrated Control of Aspergillus flavus and Aflatoxin in the Midwest Corn Belt."

An Update on Breeding Peanut for Resistance to Preharvest Aflatoxin Contamination. C. C. Holbrook[1], D. M. Wilson[2], and M. E. Matheron[3]. [1] USDA-ARS, Coastal Plain Exp. Sta., Tifton, GA; [2] Dept. of Plant Path., Univ. of GA, Tifton, GA; [3] Dept. of Plant Path., Univ. of AZ, Sommerton, AZ.

The objective of this research program is to identify sources of resistance to preharvest aflatoxin contamination (PAC) and to use these sources to develop resistant peanut cultivars. To facilitate the identification of resistant genes, a core collection was selected to represent the entire germplasm collection for peanut.

All accessions in the peanut core collection are first examined in a preliminary screen using five replications in a single environment. Genotypes which have low contamination levels in the preliminary screen are then examined for a second year using ten replications in two environments. To date, over 95% of the core collection has been examined in a preliminary screen. Of the accessions which have been tested in at least two environments, 54 continued to show low levels of resistance. Of the accessions which have been tested for at least three years, sixteen exhibited low levels of aflatoxin contamination.

Previous research has indicated a possible link between low linoleic acid content in peanut and low PAC. We conducted several studies in 1994 to examine this relationship. Due to unusually wet environmental conditions the aflatoxin contamination in all these tests was low and the results were inconclusive.

We have previously documented drought tolerance and reduced levels of PAC in six peanut plant introductions with extensive root systems. We recently observed a significant positive correlation between leaf temperature and PAC and between visual stress ratings and PAC. It may be possible to use leaf temperature and/or visual stress ratings to indirectly select for resistance to PAC. Aflatoxin contamination is an expensive trait to measure. The use of leaf temperature or visual stress ratings for preliminary screening of breeding populations for resistance to PAC would greatly reduce the expense of developing resistant cultivars.
Many of the lines which we have identified as having lower PAC have less than acceptable agronomic characteristics. These lines have been entered into a hybridization program to combine resistance to PAC with acceptable agronomic performance. We are also intermating the resistance lines. The objective of this is to combine different genes for resistance to produce genotypes with even higher resistance to PAC.

Searching for Resistance to Aflatoxin Production in Mutants of Elite Corn Inbreds

C. A. Martinson and D. R. Buxton
Iowa State University and USDA-ARS
Departments of Plant Pathology and Agronomy
Ames, IA 50011

Resistance to aflatoxin production is a desired trait in those crops where aflatoxin has become a problem. In corn there have been several instances of germplasm being developed with resistance to fungal development on the grain, but there are no examples of elite genotypes that inhibit aflatoxin production in spite of being infected by the fungus *Aspergillus flavus*. Hexane extracts of corn seed appear to inhibit aflatoxin synthesis with no apparent inhibition of growth of *A. flavus* when bioassayed *in vitro*. This may be due to some constitutive inhibitor in corn grain, and thereby a trait that can be enhanced in corn germplasm, possibly through mutagenesis. Bioassays of the *A. flavus* resistant corn germplasm developed in Georgia, Illinois, and Mississippi revealed that seed from these lines were no more inhibitory to aflatoxin synthesis than common inbred lines of corn.

A bioassay procedure was used to select for mutants in mutagenized B73 and A632 (source inbred lines for many of the current corn belt hybrids) that produce high levels of inhibitors of aflatoxin synthesis. The procedure involves adding hexane extracts to an agar medium that will allow for quantitative spectrofluorometric measurements of aflatoxin production after seeding the agar with *A. flavus*. More than 8000 M3 families from B73 and A632 have been screened and we have four families from A632 and two families from B73 that have strong inhibition of aflatoxin synthesis. The test is a destructive test and residual seed must be used to increase the germplasm. The frequency of M4 progeny with high resistance to aflatoxin synthesis varies from 2/14 to 5/14. The efforts are now being directed towards transferring this resistance to non-mutangenized A632 and B73 inbred lines. Additional families with putative aflatoxin resistance factors are being assayed to see if families can be found that have a higher frequency of resistant progeny.

The elite mutagenized B73 and A632 germplasm being used in this research was developed originally by Allen D. Wright when he was associated with the USDA-ARS at Iowa State University.

INTEGRATING FUNGAL PATHOGEN AND INSECT VECTOR RESISTANCE FOR COMPREHENSIVE PREHARVEST AFLATOXIN CONTROL IN ALMOND.

Tom Gradziel, Abhaya Dandekar, Miguel Alhumada, and Naomi Hirsch. -University of California, Davis, CA 95616
John Driver and Archie Tang. -Dry Creek Laboratories, Modesto, CA

The domestic and export almond markets presently demand zero to very low levels of aflatoxin contamination. While aflatoxin contamination in almond is relatively infrequent, when present it can occur at very high concentrations, making it a a serious health and economic problem. To achieve the necessary high levels of control we are attempting to integrate multiple genetic mechanisms for control of *Aspergillus spp.* fungi as well as Navel orangeworm (*Paramyelois transitella* Walk.) which appears important for initial fungal infection.

Resistance to fungal colonization/contamination is being pursued through the incorporation of seed coat resistance to infection, through seed based antibiosis to fungal growth, and through development of seed composition not supportive to toxin production. Genetic sources demonstrating high levels of seed coat resistance to fungal infection over the range of environments common with present production systems have been identified and are being introgressed into improved horticultural almond types. Genotypes demonstrating suppressed fungal growth and/or suppressed toxin production in seed tissue have been identified but the response appears variable depending upon the environment.

Resistance to Navel orangeworm infestations is being pursued through the development of a well-sealed endocarp, and through the development of genotypes with either antibiosis or nonpreference in the hull and/or seed tissue. Germplasm demonstrating resistance to Navel orangeworm infestation through the formation of a well-sealed yet horticulturally acceptable (high crack-out, etc.) endocarp has been identified in the related wild species *Prunus argentea*. Following three generations of backcrossing, this trait continues to show bóth good heritability and resistance. Genetic sources showing both antibiosis and nonpreference in seed and hull material have been identified, with the cultivated variety 'Mission' presently demonstrating the greatest promise. Response to general selection is low, however, and research continues on the identification of the responsible chemical agent(s) [in collaboration with USDA, Albany, CA]. Three volatiles present in mature hull tissue: carvomethenol, linalool, and gamma deca-lactone, have elicited strong nonpreference response from first instar Navel orangeworm larvae.

In order to fully exploit emerging biotech approaches to disease and pest antibiosis we have focused on the development of procedures for the transformation and regeneration of established almond cultivars, particularly the principal variety 'Nonpareil'. Goals for 1995 were the development of vigorous and disease free shoot tip cultures for transformation attempts, transformation of 'Nonpareil' tissue using *Agrobacterium* or particle bombardment approaches, and the development of adventitious shoot regeneration procedures for this very recalcitrant genotype. Clean and vigorous 'Nonpareil' tissue cultures have been established using specialized media and subculturing techniques. Transgenic leaf disk calli has been recovered using standard *Agrobacterium* procedures. Transformation has been demonstrated by expression of the GUS reporter gene and successful culture on high stringency (50mg/L) kanamycin media. Recently, adventitious shoots formation from non-transformed 'Nonpareil' callus has been achieved through specialized media/growth conditions. Work is now underway to attempt transgenic shoot regeneration from transformed calli, as well as improve the overall efficiency of the transformation/regeneration process.

USE OF A NORSOLORINIC ACID PRODUCING ASPERGILLUS PARASITICUS MUTANT TO IDENTIFY RESISTANCE TO PREHARVEST AFLATOXIN CONTAMINATION.

David M. Wilson and Neil W. Widstrom, University of Georgia and USDA/ARS, Coastal Plain Station, Tifton, GA 31793.

Aflatoxin syntheses in the norsolorinic acid (nor) producing A. parasiticus mutant, ATCC 24690, is partially blocked. When corn is wounded inoculated the fungus infects corn and produces both nor and aflatoxin. Many infected kernels can be visually identified because of the reddish color of nor. In 1994, eight corn entries were wound inoculated 20 days after full silk. Ears were rated for the number of red kernels and aflatoxin content at harvest 60 days after full silk. The red kernels occurred randomly on the ear and were not associated with the inoculation site or insect damage. There was a highly significant correlation between aflatoxin content and number of red kernels. Resistance was highly related to both the paucity of red kernels and low aflatoxin content. The use of this mutant may prove useful in developing breeding strategies for resistance to the A. flavus group and aflatoxin contamination.

Further genetic studies and progress on resistance to aflatoxin production in corn

D. G. White, T. R. Rocheford, B. Kaufman, and A. M. Hamblin
University of Illinois, Urbana, Illinois

Many dent corn hybrids currently grown in the United States use a relative of the corn inbred B73 as one parent. Inbred lines related to Mo17 are commonly used in combination with B73 type inbreds in much of the midwestern United States. Our major objective has been to identify inbred lines that contain genes that could be used to improve B73 and/or Mo17 type inbreds for resistance to Aspergillus ear rot and aflatoxin production. At the University of Illinois, an inoculation technique to screen for resistance in-field experiments was developed (3). In the summer of 1991, 1,189 F_1 hybrids with Mo17 and 978 F_1 hybrids with B73 were inoculated and evaluated for resistance to Aspergillus ear rot. From that study, 18 F_1 hybrids with B73 and 17 F_1 hybrids with Mo17 were selected for further study (2). Sources of resistance include some that were effective in Mississippi in the drought that occurred in 1993 (5). Some sources of resistance also have been shown to be resistant in laboratory studies (1, 4, 7).

From 1992 until present we have been studying the inheritance of resistance to Aspergillus ear rot and aflatoxin production in inoculated field studies. Nine sources of resistance have been studied using a generation mean analysis mating design to determine the inheritance of resistance in crosses of resistant inbreds with B73 and\or Mo17. Generation mean analysis indicated additive and dominance gene action were of primary importance in resistance to Aspergillus ear rot. With some sources of resistance, dominance genetic effect estimates were high. Inbred lines Tex 6, LB31, CI2, and Oh513 consistently had the highest levels of resistance. Frequency distributions of aflatoxin content of ears on F_2 plants and of ears on F_3 families of the Mo17 x Tex 6 and B73 x LB31 populations were highly skewed towards the resistance parent, indicative of genetic dominance. The F_2 and F_3 generations of many different crosses indicated various levels of transgressive segregation for resistance to Aspergillus ear rot and for aflatoxin production. Based on significant genetic additive and dominance effects in generation mean analysis, moderate to high heritabilities, and low estimates of effective factors, selection for resistance to Aspergillus ear rot and aflatoxin production should be effective. The frequency distribution of ear rot ratings and aflatoxin production of F_3 families and of backcross to the susceptible self families of Mo17 x Tex 6 and B73 x LB31 indicate resistant inbreds can be developed for use in commercial hybrids. We have refined an ELISA technique which allows us to do several thousand aflatoxin assays each year. To save cost on aflatoxin assays, a line development program would first select for low ear rot rating then the more resistant selections assayed for aflatoxin. The selection process could be further enhanced if RFLP markers were associated with specific genes for resistance.

Literature Cited

1. Brown, R.L., T.E. Cleveland, K.W. Campbell, and D.G. White. 1993. A comparison of postharvest resistance to aflatoxin contamination with preharvest resistance in forty-five maize genotypes. (Abstr.) Phytopathology 83:1354-1355.
2. Campbell, K.W., D.G. White, J. Toman, Jr., and T.R. Rocheford. 1993. Sources of resistance in F_1 corn hybrids to ear rot caused by *Aspergillus flavus*. Plant Dis. 77:1169.
3. Campbell, K.W., and D.G. White. 1994. An inoculation device to evaluate maize for resistance to ear rot and aflatoxin production by *Aspergillus flavus*. Plant Dis. 78:778-781.
4. Brown, R.L., T.E. Cleveland, G.A. Payne, C.P. Woloshuk, K.W. Campbell, and D.G. White. 1995. Determination of resistance to aflatoxin production in maize kernels and detection of fungal colonization using an *Aspergillus flavus* transformant expressing *Escherichia coli* β-glucuronidase. Phytopathology 85:983-989.
5. Campbell, K.W., and D.G. White. 1995. Evaluation of corn genotypes for resistance to Aspergillus ear rot, kernel infection, and aflatoxin production. Plant Dis. 79:1039-1045.
6. Campbell, K.W., and D.G. White. 1995. Inheritance of resistance to Aspergillus ear rot and aflatoxin in corn genotypes. Phytopathology 85:886-896.
7. Huang, Z.Y., D.G. White, and G.A. Payne. 1995. Inhibitory compounds to aflatoxin biosynthesis from corn line Tex6. (Abstr.) Phytopathology 85:(In press).

Chromosome Regions Associated with Resistance To Aspergillus flavus and Inhibition of Aflatoxin Production In Maize

Preharvest infection of maize (Zea mays L.) by Aspergillus spp. is a major concern to corn producers and consumers. Aflatoxins produced by Aspergillus spp. are potent toxins and carcinogens to livestock and human, and the fungal growth causes ear rot. Our objective has been to identify chromosomal regions associated with resistance to *Aspergillus flavus* ear rot and inhibition of aflatoxin production through RFLP analysis. These chromosomal regions could then be introgressed with marker assisted selection to improve commercially important lines. Various maize lines were screened for resistance to *Aspergillus flavus* and aflatoxin production. Three lines, R001, LB31 and Tex6 were identified as the better sources for resistance, and three F_3 mapping populations were developed:
R001 * B73 (96 families), LB31 * B73 (98 families), Tex6 * Mo17 (210 families). Replicated field evaluations of ear rot and aflatoxin production of the F3 Families were performed during 1992, 1993, 1994. RFLP analysis of the F3 families was conducted according to standard procedures. We probed RFLP markers distributed throughout the genome to relate marker classes to field phenotypic means.

Single factor analysis of variance has revealed several chromosomal associations with resistance to *Aspergillus flavus* and aflatoxin production. Notably, some chromosome regions have been associated with resistance for more than one source, which provides independent supportive evidence. Significant associations with either Aspergillus infection, or aflatoxin level inhibition were detected on eight chromosomes, however, major clusters of significant probes occur on chromosomes 2, 4 and 9. Significant associations for Aspergillus ear rot and not for aflatoxin level, and vice versa, (for example probes N242 and U5; U125, respectively, all on chromosome 2 in LB31* B73 families) suggests that these two traits may be partially under separate genetic control. However some chromosomal regions do appear to be associated with both traits (for example, U34 on chromosome 2, U158 on 4 and U95 on 9 in LB31* B73 families). Some significant chromosomal regions associated with Aspergillus ear rot and aflatoxin resistance are not common in the different mapping populations, suggesting there are different genes for resistance in these different resistance sources.

A recent review (McMullen & Simcox, 1995)) provides evidence for chromosomal clustering of different types of resistance genes in maize, notably some of our better associations with resistance occur in these same chromosomal regions.

The Identification of Maize Kernel Resistance Traits Potentially Useful in Commercial Germplasm Using a Kernel Screening Assay.

Brown[1], R. L., Cleveland[1], T. E., Zeringue[1], H. J., Russin[2], J. S., Guo[2], B. Z., Widstrom[3], N. W., White[4], D. G., Payne[5], G. A., Woloshuk[6], C. P.
[1]USDA/ARS/SRRC, New Orleans, La; [2]Louisiana State University, Baton Rouge, La.; [3]USDA/ARS/IBPMRL, Tifton, Ga.; [4]University of Illinois, Urbana, Il.; [5]North Carolina State University, Raleigh, NC; [6]Purdue University, West Lafayette, In.

A kernel screening assay (KSA) has been developed which facilitates quick and consistent screening of maize genotypes for resistance to aflatoxin production. The relationship between laboratory findings, using this assay, and field inoculations appears to be strong. This was demonstrated in a KSA study of kernel resistance in maize breeding population GT-MAS:gk and also in a study of 31 Illinois inbreds. This tool combined with a fungal tester strain, an Aspergillus flavus strain transformed with the B-D-glucuronidase (GUS) reporter gene linked to the B-tubulin (growth specific) gene promoter, allowed for visual and quantitative assessment of fungal growth in maize kernel tissues of several of these inbreds, and comparisons with aflatoxin production in the same kernels. Genotypes with kernel pericarp and subpericarp resistance to aflatoxin production have been identified using these tools. Pericarp resistance to aflatoxin production in genotype GT-MAS:gk has been attributed partly to its thicker wax layer. Investigations of qualitative differences between GT-MAS:gk wax and the wax of susceptible genotypes are being undertaken. Maize kernel volatiles were observed by gas chromatography/mass spectrometry. In certain resistant genotypes, the production of kernel volatiles and their relationship to fungal growth and aflatoxin inhibition was demonstrated. A study of the effects of preincubation on aflatoxin levels in resistant and susceptible kernels has provided the basis for comparing changes in the kernel physiological state with protein profile changes. This may aid in the identification of biochemical markers that are associated with resistance.

REFERENCES

Brown, R.L., P.J. Cotty, T.E. Cleveland, and N.W. Widstrom. 1993. The living maize embryo influences accumulation of aflatoxin in maize kernels. J. Food Prot. 56:967-971.

Brown, R.L., T.E. Cleveland, G.A. Payne, C.P. Woloshuk, K.W. Campbell, and D.G. White. 1995. Determination of resistance to aflatoxin production in maize kernels and detection of fungal colonization using an Aspergillus flavus transformant expressing Escherichia coli B-glucuronidase. Phytopathology 85:983-989.

Guo, B.Z., J.S. Russin, T.E. Cleveland, R.L. Brown, and N.W. Widstrom. 1995. Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by Aspergillus flavus. J. Food Prot. 58:296-300.

Tubajika, K.M., B.Z. Guo, J.S. Russin, R.L. Brown, T.E. Cleveland, and N.W. Widstrom. 1995. Factors associated with resistance to aflatoxin production in maize. Phytopathology 85:512. Abst.

Guo, B.Z., J.S. Russin, R.L. Brown, T.E. Cleveland, and N.W. Widstrom. 1995. Resistance to aflatoxin contamination in maize influenced by relative humidity and kernel germination. J. Food Prot. (In press).

Zeringue, H.J., R.L. Brown, J.N. Neucere, and T.E. Cleveland. 1994. Relationships between C6-C12 alkanal and alkenal volatile contents and resistance of maize genotypes to Aspergillus flavus and aflatoxin production. Proceedings of the USDA-ARS Aflatoxin Elimination Workshop, St. Louis, MO. Abst.

Temporal Interactions of *Aspergillus* Infection and Aflatoxin Accumulation in Corn

Gary L. Windham, Research Plant Pathologist, and W. Paul Williams, Research Geneticist, USDA-ARS, Crop Science Research Lab, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

Infection of maize kernels by *Aspergillus flavus* and subsequent accumulation of aflatoxin is a chronic economic problem in the southeastern United States and can be a problem in the Corn Belt. The etiology of *A. flavus* infection in maize kernels and onset of aflatoxin production is not very well defined. Information on the timing of kernel infection and aflatoxin accumulation is needed to help researchers determine when to evaluate maize genotypes for resistance.

Field studies were conducted for two years at the Plant Science Research Center, Mississippi State, MS, to monitor maize kernel infection and aflatoxin accumulation during the growing season. Also, hybrids resistant and susceptible to *A. flavus* were compared to determine differences in infection and aflatoxin levels. The resistant hybrids included Mo18WxMp313E, Mp420xTx601, and SC54xSC76; the susceptible hybrids included GA209xMp339, Mp307xMp428, and Mp68:616xSC212M. Ears were inoculated at 7 days after midsilk using the side needle technique. The top ear of each plant was inoculated with a 3.4 ml suspension containing 3 X 10^8 *A. flavus* conidia. Inoculated ears were harvested at 35, 42, 49, 56, and 63 days after midsilk. Grain moisture was determined at each harvest date. Kernel infection was determined by plating kernels on Czapek agar amended with 7.5% NaCl. Aflatoxin levels were determined by using the Vicam "Aflatest Procedure".

Differences between resistant and susceptible hybrids for aflatoxin levels were observed on the first harvest date (35 days after midsilk). Aflatoxin levels ranged from 26 ppb for Mp420xTx601 to 97 ppb for GA209xMp339. Significant differences between resistant and susceptible hybrids for kernel infection were not observed until 42 days after midsilk. There were also differences between resistant and susceptible hybrids in kernel infection and aflatoxin levels at 49, 56, 63 days after midsilk. Kernel infection (8.1% for GA209xMp339) was highest at 49 days after midsilk, and aflatoxin levels (510 ppb for Mp307xMp428) were highest at 63 days after midsilk. The relationship between days after midsilk to aflatoxin accumulation was best described by linear models for all of the hybrids. Maximum differences between resistant and susceptible hybrids for aflatoxin levels were observed at 63 days after midsilk. Two of the resistant hybrids, Mo18WxMp313E and Mp420xTx601, had significantly less aflatoxin than the three susceptible hybrids at 63 days after midsilk. Although grain moisture was negatively correlated with aflatoxin levels, little difference in moisture between resistant and susceptible hybrids was observed.

Identifying Heat Tolerant Sources of Corn Germplasm with Reduced Susceptibility to Aflatoxin Contamination

James R. Dunlap, Associate Professor, Texas A&M University System, Texas Agricultural Experiment Station, Weslaco, Texas 78596

Over 150 hybrids have been tested in South Texas at the Weslaco location during the last 5 years in order to identify corn hybrids and environmental effects on the developing kernel that may be associated with reduced susceptibility to aflatoxin contamination. Reproducible decreases in aflatoxin contamination have been obtained with several tropical hybrids obtained from DeKalb Genetics and Ceres Seed. These hybrids seem to express a greater tolerance to high temperatures and humidity typically encountered in South Texas and the southeastern United States. The kernels appeared fuller and yields were approximately 25% greater than other domestic hybrids tested in 1995. Eight of the hybrids were identified as less susceptible by not exceeding a threshold of 400 ppb aflatoxin in the inoculated tests. This is in contrast to the "susceptible" hybrids that accumulated up to 2500 ppb in the same tests. Most of the tropical germplasm including the less susceptible hybrids developed a tight husk. The tight husk conferred protection despite inoculating through the husk cover. Tests using *in vitro* kernel cultures imply that the pericarp must be broken if contamination is to occur. Using kernel culture techniques and hybrids expressing a decreased susceptibility offers new opportunities to understand the kernel-based mechanisms responsible for susceptibility to aflatoxin contamination. It also is obvious that heat tolerant tropical hybrids offer new choices for producers operating in regions of the U.S. where the risk of aflatoxin outbreaks is high.

Understanding the Structure/Function of PR-5 Anti-Fungal Proteins

R.A. Bressan, D.-J. Yun, L. Todd, M. Narasimhan, Y. Zhao, J. Pardo, P.M. Hasegawa.
Department of Horticulture, Purdue University, 1165 Horticulture Bldg., W. Lafayette, IN 47907-1165

Almost all of the recently discovered antifungal proteins exhibit very low levels of activity against *Aspergillus* sp. Our recent studies with some of the PR-antifungal proteins has indicated that these proteins exhibit their activity in a target-specific manner. We have begun to study the structural and genetic determinants that control the ability of PR-5 proteins to target specific fungi. We have determined that the antifungal activity of PR-5 proteins is controlled by factors present on both the cell wall and plasmamembrane of target fungi. Recently, we have cloned several genes from yeast that control PR-5 sensitivity. Homologues of some of these genes are present in other fungi and may function to control the sensitivity of many fungi including *Aspergillus* to PR-5 proteins. It is expected that detailed knowledge of the mechanisms of PR-5 protein activity against fungi and the defenses used by recalcitrant fungi like *Aspergillus* will allow us to genetically engineer much more active forms of these proteins.

CROP RESISTANCE - GENETIC ENGINEERING

PANEL DISCUSSION TITLE: Genetic Engineering for Resistance: New Opportunities.

PANEL MEMBERS: Ed Cleveland (Chair), Jeff Cary, Caryl Chlan, Peggy Ozias-Akins, Arthur Weissinger, Ray Bresson and Mary Lou Mendum.

SUMMARY OF PRESENTATIONS: Speakers presented their progress in genetic engineering of crops subject to aflatoxin contamination (cotton, walnut and peanut) to enhance resistance in the crops to A. flavus invasion. Various examples of possible antifungal genes effective against A. flavus were presented by the speakers including those encoding osmotin, chitinase, chitin binding proteins, polygalacturonase inhibitor protein and lytic peptides (cecropin analogs). Results also were presented demonstrating lytic activity against A. flavus of a new peptide being supplied through an agreement between Demeter Biotechnologies Ltd., the Agricultural Research Service (USDA) and University cooperators. Bioassays involving certain of these recently available lytic peptides have shown that they are particularly effective against young germinating conidia of A. flavus.

SUMMARY OF PANEL DISCUSSION: As in previous workshops, sources of effective antifungal genes was one of the topics of discussion. In the past, the lack of gene encoded products which are directly toxic to A. flavus has caused great concern among the various laboratories engaged in genetic engineering of peanut, cotton and tree nuts. The reason for this concern involves the considerable effort that goes into production of crops stably transformed with foreign genes, and thus, it is highly desirable to work with genes that have the highest potential for limiting fungal growth and/or aflatoxin contamination. However, confidence is obviously building within the "crop genetic engineering group" that viable anti-A. flavus genes do indeed exist. This was quite evident during the panel discussion since the topic of identifying candidate antifungal genes did not dominate the discussions as it did in previous years.

The possibility of increased communication and sharing of gene regulatory elements and vector constructs by members of the plant genetic engineering group was discussed. An increased sharing of genetic materials would be mutually beneficial since a number of antifungal genes along with regulatory elements to yield tissue specific expression have been cloned into plant transformation vectors by the various laboratories. These constructs may have generic value for genetic engineering of cotton, tree nuts or peanut. There was broad agreement that no single antifungal gene is likely to provide complete protection against A. flavus. For this reason, it was suggested that the group coordinate the construction of transformation vectors carrying two or more antifungal genes. Through a coordinated effort, it would be possible to produce a significant number of multi-gene constructs which could be pooled among workers for test in the various

crops. The suggestion was made that it would be useful to have a rapid method for assessing tissue specific expression of constructs, perhaps through visualization of transient expression in specific plant cell/tissue types. However, it was also mentioned that if specific plant tissues are substantially modified during the transformation protocols, they could lose their tissue "identity" and thus will not yield authentic tissue-specific regulation. Despite these uncertainties, significant hope exists in the fact that many promoter elements from crops representing highly divergent plant families demonstrate authentic tissue specific regulation even when engineered into heterologous hosts. For example, a seed storage protein gene promoter from cotton was shown to be specifically recognized and to endow certain genes with seed specific expression in tobacco.

Another topic that was introduced for discussion by the panel chair was the use of resistance genes/traits identified by researchers presenting results in other Workshop sessions. The question should be asked on a continual basis as to whether better candidate genes, no matter what the source, are available for genetic engineering of crops for resistance to A. flavus. For example, a number of studies were presented by plant pathologists and plant breeders during the Workshop session entitled "Crop Resistance-Conventional Breeding" which indicated that active resistance chemicals exist in kernel tissues. One possible candidate that was mentioned was glucanase, an enzyme which exists in corn kernels and which can be lytic to A. flavus. Also, workshop participants in the special poster session entitled "Fundamental Genetic Mechanisms Controlling Aflatoxin Biosynthesis" reported on the identification of factors in corn and soybean that could potentially limit A. flavus growth and/or aflatoxin biosynthesis; these chemical factors could represent useful sources of genes for genetic engineering of plants. The point was well taken that laboratories conducting plant genetic engineering could take advantage of knowledge being produced by scientists in other workshop sessions.

Finally, one topic that stimulated significant discussion between the audience and panel members was the concept of stimulating "natural" resistance within crops vulnerable to aflatoxin contamination. For example, genes governing systemic acquired resistance (SAR) have been cloned and perhaps could be utilized in genetic engineering of peanut, cotton or tree nuts for enhancement of resistance to A. flavus invasion. However, most of the studies involving SAR have focussed on induction of resistance against vascular system or foliar invading pathogens and little is known about induction of SAR in the fruit. More work will be needed to establish if SAR is functional not only in vegetative tissues but also in fruits and seeds of crops contaminated by aflatoxin.

Gene Constructs Encoding A. flavus Growth Inhibitors for Genetic Engineering of Cotton, Peanut, and Tree Nuts. Jeffrey W. Cary, Anthony DeLucca, Thomas E. Cleveland, USDA/ARS, SRRC, New Orleans, LA 70179; Caryl Chlan, University of Southwestern Louisiana, Lafayette, LA 70504.

Over the past year our group has continued it's efforts in the construction and transfer into cotton of vectors harboring genes that will impart increased resistance to *A. flavus* invasion and hence aflatoxin contamination. Our initial approach has been to use a basic vector construct consisting of the potential antifungal gene under the control of the constitutive CaMV 35S promoter with a selectable kanamycin (Km) resistance gene marker also present. Utilizing biolistic transformation techniques we have delivered into cotton, vector constructs harboring genes producing potential antifungal proteins such as bean chitinase, tobacco osmotin, potato protease inhibitor II (PPI II), and bean polygalacturonase (PGIP) inhibitors. Numerous Km resistant F_0 cotton plants have been regenerated and F_1 progeny recovered from these primary transformants. All are currently being analyzed for the presence and expression of their respective antifungal genes.

Recently we have turned our attention to the efficacy of small lytic peptides (e.g. cecropins, defensins, and magainins) as inhibitors of growth of *A. flavus*. Due to their impressive antimicrobial activity at low concentrations and small size these lytic peptides are excellent candidates as antifungal agents and readily lend themselves to genetic manipulation. In addition, because these peptides are quite small in size (in general ≤39 amino acid residues) and composed of standard amino acids, they can be readily synthesized in quantities sufficient for performing extensive *in vitro* analyses. Through our collaboration with Demeter Biotechnologies, Ltd. we have obtained a lytic peptide designated DAFM (Demeter Antifungal Molecule) for analysis of it's efficacy as an inhibitor of growth of *A. flavus*. Our *in vitro* assays determined the efficacy of DAFM vs. cecropin against *A. flavus* and the fungal pathogen and mycotoxin producer, *Fusarium moniliforme*. Results of this work have shown that with *A. flavus*, DAFM and cecropin give 50% inhibition of hyphal growth at concentrations of ~5-10 μM, while 100% inhibition was achieved at a concentrations of 12.5 μM and 25 μM for DAFM and cecropin respectively. Germination of *A. flavus* spores was not inhibited by either of the peptides. Interestingly, both spore germination and hyphal growth of *F. moniliforme* were inhibited greater than 95% at concentrations of ~2-3 μM DAFM or cecropin. Experiments to determine this large difference in sensitivity of *A. flavus* compared to *F. moniliforme* to these peptides has indicated a correlation between sensitivity and levels of the sterol, ergosterol, a lipid present in fungal spores and hyphae. Previous work with gram negative bacteria has shown that cecropin has an affinity for the lipid portion of lipopolysaccharides of the outer membrane. Sedimentation analysis and fluorescence microscopy have shown that cecropin preferentially binds to ergosterol and *F. moniliforme* spores contain up to 10X more ergosterol than is found in *A. flavus* spores. We have theorized that DAFM may also have an affinity to ergosterol similar to that of cecropin.

A critical factor in the *in vivo* efficacy of these peptides as antifungal agents will be their susceptibility to protease digestion. We have conducted studies on the sensitivity of these peptides to degradation by proteases elicited by the fungus as well as the cotton plant. Results of fungal protease assays have shown that cecropin is extensively degraded by *A. flavus* proteases within 2 h while DAFM is virtually unaffected. Cotton leaf proteases present in the intercellular fluids completely degraded cecropin within 30-45 min. DAFM was also

degraded but in a limited fashion, as after 2 h a significant quantity of one peptide species remained that represented DAFM with only it's carboxy-terminal residue removed as determined by mass spectroscopy. Further studies are being performed to determine if this DAFM degradation product is still capable of inhibiting *A. flavus* growth. These results bode well for the potential of DAFM as an inhibitor of *A. flavus* growth *in vivo*. We have constructed two plant transformation vectors harboring a synthetic gene coding for DAFM. One places the DAFM gene under control of the CaMV 35S promoter while the other has DAFM translationally fused to a potato ubiquitin gene under the control of the constitutive ubi3 promoter. These constructs have been delivered into cotton via biolistic transformation and we are awaiting production of enough regenerated plant tissue to begin analysis for presence and expression of the gene and bioassay of transgenic plants. We will also be placing the DAFM gene and others under the control of seed-specific and wound-inducible promoters. We have used constructs consisting of a GUS reporter gene under control of either of the wound-inducible promoters, ubi7 or PPI II from potato, in transformation experiments to determine their expression profiles in cotton.

Title: Biolistic Transformation of Cotton to Generate Increased Resistance to A. flavus

Authors: Caryl A. Chlan, Manjula Panati, and Lin Junmin (Biology Department, The University of Southwestern Louisiana, Lafayette, LA 70504) and Jeffrey Cary and Thomas E. Cleveland (Southern Regional Research Laboratory, USDA/ARS, New Orleans, LA 70179)

Our goal is to transform and regenerate cotton plants that have been genetically engineered in the laboratory to express genes that confer increased resistance to Aspergillus flavus. Cottonseed is an important by product of the cotton industry, and its monetary value as animal feed is affected by aflatoxin levels. Because conventional control measures have not been effective, and strains of cotton that are naturally resistant to A. flavus are not available, genetic engineering of cotton at the molecular level is a logical approach to this problem.

We are using a biolistic method to transform cotton meristem tissues. Our optimized conditions include sterilization of seeds, dissection of meristem tissue, followed by bombardment with optimized parameters, and subsequent regeneration of plants. Our protocol includes bombardment of the tissue the same day that it is dissected with DNA coated 1.6 micron gold particles accelerated by pressures of 1300 or 1550 psi at tissues a distance of 6 cm. Transformation efficiency is also increased if the same tissue is bombarded twice (either both times at a distance of 6 cm, or once at 6 cm then once at 3 cm). After the tissue has been bombarded, the sections are placed on McCown's media supplemented with kanamycin at 50 mg/L. Actively growing sections are excised from dead tissue, and placed on fresh McCown's plus kanamycin as necessary. The plants that root in the presence are then transferred to a special rooting medium to favor formation of an adequate root system. Using two different expression assays, these plants test positive for Neomycin Phosphotransferase II. Our first generation of potential anti-fungal gene constructs included DNA that encode NPT II and a potential anti-Aspergillus flavus activity - either osmotin, chitinase, polygalacturonidase inhibitor protein or a proteinase inhibitor. Our "second generation" of potential anti-fungal gene constructs include a chitinase/glucanase dual construct, a Demeter anti-fungal gene construct, and an osmotin gene dual construct. These genes have been used to bombard cotton meristem tissue and the tissues regenerated as described above.

We have begun testing plants that express NPT II activity for the presence and expression of the anti-fungal genes. To determine if the anti-fungal gene is present, DNA is extracted from leaf tissue, and subsequently analyzed using the Polymerase Chain Reaction. Using this approach, we have identified a cotton plant that appears to contain osmotin DNA sequences. When DNA from this plant is amplified using osmotin specific primers, a specific DNA fragment is formed that cross hybridizes with an osmotin probe on a Southern blot. DNA from several F_1 progeny of this plant have been tested for osmotin sequences with PCR. A putative F_1 has been identified, however we have not yet confirmed this observation using other approaches. If this F_1 contain the osmotin gene, then we will have successfully generated a germ line transformant. We are in the process of analyzing the leaf tissue of this plant for the presence of osmotin protein using western blot analysis, and plan to test the DNA for the presence of the osmotin sequences with Southern blot analysis in the near future. We have two additional putative osmotin transformed cotton plants from which leaf protein extracts cross react with anti-osmotin antibody on western blots. We are in the process of confirming the presence of the osmotin gene in these plants with PCR and Southern analyses. After we have identified plants that contain the genes to enhance resistance to Aspergillus flavus, and express those genes in detectable quantities, plant extracts will be tested for their ability to inhibit the pathogen.

GENETIC ENGINEERING OF PEANUT - PROGRESS WITH Bt AND FUNCTION OF A SOYBEAN PROMOTER IN PEANUT

Peggy Ozias-Akins, Hanli Fan, and Aiming Wang
Department of Horticulture, University of Georgia Coastal Plain Experiment Station, Tifton, GA

Our transformation protocol using microprojectile bombardment of embryogenic tissues has now been refined to 1) allow a complete plant cycle from initiation of embryogenic cultures, through transformation and regeneration, to production of numerous pods in 18 months or less, 2) reduce the total time required for the transformation process and production of pods to approximately 12 months, 3) increase the efficiency of recovery of transgenic cell lines approximately 25 fold relative to our first experiments.

The most critical factor for recovery of fertile transgenic plants using our transformation system is culture age. Most lines recovered from experiments initiated with cultures 10 months old or less are fertile and produce numerous pods. Existing transgenic plants containing the Bt or peroxidase genes were obtained from much older cultures and most apparently are not fertile, although progeny have been obtained from one Bt-positive plant. The progeny will be tested for expression of Bt both by ELISA and bioassay.

Our most successful experiments have been with the insertion of a promoter-GUS construct. The promoter is from a soybean vegetative storage protein gene (*vsp*). This gene in soybean is inducible by wounding, methyl jasmonate, carbohydrates, and water deficit. The vegetative storage protein accumulates to high levels in immature soybean pod walls. When the *vsp* promoter-GUS gene fusion is inserted into peanut, expression of the gene follows expected temporal and spatial patterns as would be predicted from soybean. There is lower expression in leaves than in stems. Expression in both organs increases in response to excision and treatment with jasmonic acid. Expression appears highest around the vascular tissue which probably reflects sucrose-induced expression. Expression is particularly high in young peanut pods. This pattern of expression might be significant for engineering of Bt-producing lines where our target pest is lesser cornstalk borer (LCB). LCB attacks primarily the stem and pod wall, the two locations where expression of *vsp*-GUS is highest in peanut. It might also be useful for engineering expression of broad-spectrum antifungal/antibacterial compounds such as lytic peptides. Expression of GUS controlled by this *vsp* promoter is very low to undetectable in roots where the natural symbiosis with *Rhizobium* should not be disturbed by antifungal strategies.

Transformation of Peanut cv. 'NC 7' With Genes Encoding Defensive Peptides
Arthur Weissinger [1], Rebecca Cade [1] and Lori Urban [1], in collaboration with Gary Payne [2], Peggy Ozias-Akins [3], Jesse Jaynes [4], Mike Hasegawa [5] and Ray Bressan [5]. Depts. of [1] Crop Science and [2] Plant Pathology, N. C. State University; [3] Department of Horticulture, University of Georgia Coastal Plain Experiment Station; [4] Demeter Biotechnologies, Inc.; [5] Center for Plant Environmental Stress Physiology, Purdue University

Current objectives include: improvement of peanut transformation protocols to enhance the recovery of fertile transgenic plants; transformation of peanut with a synthetic gene encoding the cecropin analog D5C (Demeter Biotechnologies, Inc.); transformation of peanut with a gene encoding the anti-fungal peptide osmotin, derived from tobacco; and the development of test strategies to measure the efficacy of defensive peptides expressed in peanut against infection by *Aspergillus flavus* and other fungal pathogens.

New protocols for culture, selection and regeneration of transgenic peanuts have been developed using peanut cv. NC 7, and have subsequently been tested on cv. Marc I. First, embryogenic cultures are initiated from embryonic axes excised from dry, mature seed, in place of the immature embryos used in earlier protocols. This allows the initiation of large numbers of cultures without the requirement for maintenance of stock plants in the greenhouse. The response frequency of this explant is comparable to that of immature embryos in cv. NC 7. Secondly, the time over which cultures are carried prior to transformation has been greatly reduced, in order to reduce adverse effects often associated with protracted tissue culture. Secondary embryos are harvested for transformation by microprojectile bombardment after 16 weeks in culture (sufficient for the initiation of secondary embryogenesis). Cultures are either exhausted or discarded after 24 weeks following culture initiation. Finally, regeneration and rooting protocols have been modified. Transformed embryos are carried for 2-4 weeks on MS basal medium with 4mg/l of benzyl aminopurine (BA) and 2mg/l naphthalene acetic acid (NAA). They are then transferred to MS basal medium with 4mg/l BA and .1mg/l NAA for an additional 4-6 weeks.). Time in liquid selection has been reduced from 6 to 4 weeks. This has resulted in a reduction of the time required for flowering following bombardment by approximately 4 weeks, without a detectable increase in the rate of escapes. Transformed plantlets are rooted on half-strength MS basal medium with 1mg/l indole acetic acid (IAA), resulting in a significant increase in rooting efficiency.

Using these protocols, 22 plants transformed with a gene encoding the synthetic cytolytic peptide D5C have been recovered from 12 independently transformed cell lines. 18 of these plants flowered within 3 months of transfer from culture to soil. Of these, 17 have pegged, and have either set seed or are currently developing pods. This level of fertility represents a very significant improvement over earlier protocols. Rooted cuttings have been prepared from transgenics for efficacy testing.

Experiments have also been initiated for introduction of the tobacco osmotin gene into peanut by both microprojectile bombardment and *Agrobacterium*-mediated DNA transfer. Preliminary data suggest that tDNA carrying the osmotin gene has been successfully transferred into cv. Valencia A.

PROGRESS IN ENGINEERING WALNUTS FOR RESISTANCE TO *ASPERGILLUS FLAVUS*

Mary Lou Mendum, Gale McGranahan, Abhaya Dandekar, and Sandra Uratsu
Department of Pomology, University of California, Davis CA 95616

Our goal is to reduce aflatoxin contamination in tree nuts by genetically engineering the host plant to resist infection by *Aspergillus flavus*. For the tree nut crops, walnuts can serve as a model system because transformation and regeneration of transgenic plants is relatively routine. The same system has been applied successfully to pecan. Additionally, the target tissue for genetic transformation in walnuts is the somatic embryo, which multiplies readily in culture and is similar to the walnut kernel. Somatic embryos can be used to assess the response of the causal fungus to the introduced genes, *in vitro*. This research summary describes our progress in 1995.

Our objectives were: 1) to begin field trials of selected clones containing chitin binding genes, 2) to confirm *via* Southern blotting incorporation of a systemic acquired resistance gene (SAR 8.2) into walnut embryo lines, and test this gene's efficacy *in vitro* against *A. flavus*, and 3) to investigate the potential of other genes for controlling *A. flavus*, including a modified *Bacillus thuringiensis* gene for control of insect vectors. (Work on this last gene is partially funded by the Walnut Marketing Board.)

Two transgenic walnut embryo lines containing chitin-binding genes (barley lectin and nettle lectin) have been shown to significantly reduce *A. flavus* sporulation *in vitro* ($p<0.004$ and $p<0.009$, respectively). These have been germinated, grown into shoots, and grafted onto seedling rootstock in preparation for field trials. Six of eight transgenic embryo lines containing the SAR 8.2 gene were tested against *A. flavus*, and did not reduce fungal sporulation *in vitro*. We have confirmed transformation of three walnut embryo lines containing a Series III synthetic *B. thuringiensis cryI A(c)* gene, with a modified codon bias and a transit peptide to target the resulting protein to the chloroplast. These lines are being bulked up to provide tissue to assay their insecticidal activity. Walnut clones containing Series II *cryI A(c)* genes, which lack the transit peptide, are currently in field trials.

Several recent technical advances have improved our ability to asses the potential of new genes introduced through genetic engineering. A new technique for cryopreservation of walnut somatic embryos is being used for long-term storage of embryo lines, to prevent somaclonal variation and loss of lines to contamination. Improvements in somatic embryo germination and grafting techniques have made it easier to produce transgenic trees for field trials. A new field trial of over 100 transgenic walnut clones representing 10 introduced genes has been approved by APHIS and is currently being planted. Finally, culture of immature anther tissue has resulted in several somatic embryo lines, opening the possibility of introducing new genes directly into elite cultivars.

CROP RESISTANCE - IDENTIFICATION OF INHIBITORS OF FUNGAL GROWTH AND/OR AFLATOXIN BIOSYNTHESIS

Differential Effects of 9- and 13-hydroperoxy fatty acids on *Aspergillus* Growth and Mycotoxin Biosynthesis.

G. B. BUROW*, T. C. NESBITT*, J. DUNLAP** and N. P. KELLER*.
*Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843-2132.
**Texas Agricultural Experiment Station, Texas A&M University, 2415 E Hwy83, Weslaco, TX 78596

ABSTRACT

Aspergillus fungi are known for their ability to contaminate food and feed crops - such as corn, peanuts, cottonseed and tree nuts - with the carcinogenic mycotoxins, aflatoxin (AF) and sterigmatocystin (ST). In an attempt to identify plant metabolites which may induce or inhibit mycotoxin biosynthesis, we have investigated the effects of lipoxygenase (LOX) products on *Aspergillus* growth and subsequent AF/ST biosynthesis. LOXs are stress-response plant enzymes which catalyze the conversion of the polyunsaturated fatty acids linoleic and linolenic acid to primarily 13-hydroperoxy fatty acids, 13-HPODE and 13-HPOTE (e.g. soybean seed LOX1) or primarily 9-hydroperoxy fatty acids, 9-HPODE and 9-HPOTE (e.g. corn seed LOX). At µM concentrations, 13-HPODE and 13-HPOTE decreased *Aspergillus* growth, inhibited ST and AF gene transcription and reduced ST and AF production by *A. nidulans* and *A. parasiticus* respectively. In contrast, the same concentration of 9-HPODE had no-to-little effect on fungal growth, AF gene expression and AF production in *A. parasiticus* under the same conditions. We suggest that hydroperoxy fatty acids and/or their metabolized products may play a important role in regulating *Aspergillus* infections and subsequent mycotoxin biosynthesis *in situ*.

Bioactivity of Sorghum Antifungal Proteins

Seetharaman, K., Whitehead, E., Keller, N. P.* and Waniska, R. D.
Dept of Soil and Crop Sciences and Dept of Plant Pathology*
Texas A&M University, College Station, TX 77843-2474

ABSTRACT

The role of sorghum antifungal proteins (AFPs) in sorghum grain molding was investigated. Several AFPs, such as sormatin, chitinases, glucanases and ribosome inhibiting proteins (RIP), have been identified in sorghums . AFPs from sorghum seeds were extracted, purified using 55% ammonium sulfate precipitation and eluted from a CM-Sephadex column using a 200-500 mM salt gradient. Fractions were tested for bioactivity against *Fusarium moniliforme*, *Curvularia lunata*, *Aspergillus flavus* and *Aspergillus parasiticus* using (a) hyphal rupture, (b) hyphal extension, and © spore germination methods.

F. moniliforme and *C. lunata*. *F. moniliforme* exhibited hyphal rupture at the growing tip and other regions of mycelium at protein levels as low as 20 μg of a fraction containing several AFPs. *C. lunata* required higher protein levels (20-100 μg) and ruptured only at hyphal tips. Spore germination in both species was completely inhibited by <100 μg protein. Fungal spores germinated when the protein fraction was boiled, suggesting the involvement of proteins.

Spore germination in both *Aspergillus* species was completely inhibited by the fraction containing several AFPs. However, spore germination was also inhibited after boiling the fraction. Both *Aspergillus* species did not exhibit hyphal disruption when treated with AFPs. Further tests are in progress to identify the source of this inhibition and the mode of action.

The strategy of using a mixture of AFPs vs. an individual AFP could synergistically increase plant resistance against several pathogens.

Aspergillus flavus Grown on Glass Fiber Filters is a Sensitive Method for Evaluating Inhibitors of Growth and Aflatoxin Production. ROBERT A. NORTON, USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

A new method of growing *A. flavus* was developed to evaluate compounds occurring in corn kernels for inhibition or stimulation of growth and aflatoxin B_1 (AFT B_1) production. The suspended disc culture system consists of a humidified vial with a septum cap pierced by a pin on which a glass fiber disc is affixed. The disc contains the medium, inoculum and test compound. The method is particularly useful for testing hydrophobic compounds without solubilizers and compounds available in very small amounts. Discs of glass fiber, quartz fiber and filter paper were evaluated for AFT B_1 production and coefficient of variation (CV). The best overall performance was obtained with glass fiber filters containing binder. AFT B_1 production is ca. 5 times greater for glass fiber discs than for liquid medium layers with equivalent surface area to volume. Discs as small as 6mm, using 8 ul of medium, had a lower CV (15%) than liquid cultures with 20-30 times more medium. Application of the method has been demonstrated with studies of corn kernel metabolites and related compounds representing very different solubility groups. 4-Acetybenzoxazolinone (4-ABOA) inhibited AFT B_1 production 35% at 0.08 mg/ml and 75% at 0.4 mg/ml with little effect on growth (4-ABOA and the following BOAs are all soluble in acetone). 6-Methoxybenzoxazolin one (MBOA) produced complete inhibition of growth and toxin production at 0.68 mg/ml, a reduction of 45% at 0.4 mg/ml but only 8.5% in growth. Benzoxazolinone (BOA) produced declines in both AFT B_1 and growth at levels of 0.016 mg/ml and 0.08 mg/ml with no growth or toxin at 0.4 mg/ml. Cyanidin (water soluble) at 5.0 and 20 mg/ml inhibited toxin by 37% and 87%. Growth was increased by 12% at 20mg/ml and decreased by 18% and 9% at 1.25 and 5 mg/ml. The steryl ferulate and *p*-coumarate fraction from corn bran (hexane soluble) either stimulated growth and aflatoxin or had no effect, depending on *A. flavus* strain (NRRL 6536 and NRRL 3357 respecively). The suspended disc method allowed testing of these compounds with a wide range of solubilities in small amounts and with acceptable variation.

EFFECT OF SELECTED CORN METABOLITES ON GROWTH AND AFLATOXIN PRODUCTION BY *Aspergillus flavus*. Robert A. Norton, USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

Contamination of preharvest corn by *Aspergillus flavus* and production of aflatoxins in contaminated grain continues to be a concern due to the health and economic losses incurred by susceptible corn lines. A number of compounds occur in corn kernels for which no studies have been done on their ability to affect *A. flavus*. The objective of the work described here was to evaluate selected metabolites in this group for their effect on growth and aflatoxin B_1 (AFT B_1) production by *Aspergillus flavus* NRRL 3357. The results would indicate if known compounds, at higher levels or greater tissue specificity, are capable of being used to control the level of aflatoxin in corn. The suspended disc culture system, which has been previously described (Norton, 1995), was used to evaluate the compounds.

Amides of putrescine and ferulic acid or *p*-coumaric acid occur primarily in the pericarp and have been reported to inhibit Fusarium graminearum (J. T. Arnason, pers. commun.). Feruloyl-, diferuloyl-, and *p*-coumaroylputrescine (FP, DFP, CP) were tested at levels of 62.5, 125, 250, 500, and 1000 μg/ml. FP produced a 10% decrease in AFT B_1 at the highest concentration with no effect on mycelium dry weight. There was no effect on toxin or weight from any level of CP. DFP produced about a 12% decrease in AFT B_1 at levels above 125 μg/ml but no had no effect on weight. Overall this group of compounds had little effect on *A. flavus*. The possibility that the putrescine compounds might enhance the inhibition of AFT B_1 produced by 4-acetylbenzoxazolinone (4-ABOA), which also occurs in the pericarp, was tested using a moderately inhibitory level of 4-ABOA (80 μg/ml) and 250 μg/ml DFP or CP. No effect on the inhibition from 4-ABOA was found.

Maysin, a luteolin-C-glycoside, is reported to occur at high levels in the cornsilk of some lines and appears to confer resistance to corn earworm (Snook et al., 1994). Maysin was tested at levels of 1, 10, 100, 1000 μg/ml. No significant effect on either growth or aflatoxin was found.

Anthocyanins (as glycosides) are responsible for red and purple coloration occurring in the aleurone or pericarp of some lines of Indian corn. The concentration of these compounds can be high within the tissue in which they occur. The aglycones cyanidin and pelargonidin were tested at levels of 1.25, 5, and 20 mg/ml. Cyanidin inhibited AFT B_1 by ca. 37% at 5 mg/ml and 93% at 20 mg/ml; growth was increased ca. 12% at 20 mg/ml. Pelargonidin had no effect on toxin at 0.31 mg/ml, inhibited ca. 68% at 1.25 mg/ml and over 99% at 5 mg/ml; growth decreased to a minimum of 63% of the control at 1.25 mg/ml.

The antioxidants α- and γ-tocopherol occur primarily in the germ in corn. Other antioxidants, e.g. butylated hydroxytoluene (BHT), have been found to inhibit aflatoxin formation in *A. parasiticus* (Lin & Fung, 1983). Both α- and γ-tocopherol were tested at 20, 63, 200, 632, and 2000 μg/ml. Neither tocopherol had a significant effect on mycelium weight. AFT B_1 was 20% higher than the control at 2000 μg/ml of α-tocopherol; γ-tocopherol had no affect on AFT B_1 level. These compounds do not appear to be a factor in AFT B_1 production.

Several carotenoids are found in the endosperm of corn, especially yellow dent lines. Two hydrocarbon representatives of this group, α- and β-carotene were tested. Zeaxanthin and lutein are two major oxygenated carotenes occurring in corn. They have identical ring

structures to the corresponding carotenes but contain a hydroxy group on each ring. The four carotenoids were tested at levels from 0.032 to 1000 ug/ml. β-Carotene and zeaxanthin had similar profiles, with inhibition of AFT B_1 beginning at 1 μg/ml and decreasing to 15% and 25% of control, respectively, at 1 mg/ml. Similarly, α-carotene and lutein had similar inhibition profiles with 40% toxin inhibition at 0.032 ug/ml and inhibition of 98% and 96%, respectively, at 1 mg/ml. There was no appreciable effect on growth at any concentration of the carotenoids. Highest inhibition was strongly correlated with the presence of the "epsilon" type ring structure in lutein and α-carotene; each compound has one epsilon ring and one beta type ring. Comparison of activity with α-, and β-ionone, which have the same ring structures, showed moderate inhibition for α-ionone and no effect from β-ionone. β-Ionone is thought to be a breakdown product of carotenes containing a beta ring.

The results of this research show that compounds capable of inhibiting aflatoxin formation exist in the kernel. However, approximately 85% of carotenoids occur in the pericarp and only small amounts in the germ. The problem with this group of active compounds is that the site of accumulation is not at the site at which most aflatoxin is found, i.e. the germ.

REFERENCES

Norton, R.A. (1995). Mycopathologia 129:103-109.
Snook, M.E. et al. (1994). ACS Symposium Ser. 557, pp. 122-135.
Lin, C.C.S and Y.C. Fung (1983). J. Food Sci. 48:576-580.

CHARACTERIZATION OF INHIBITORY COMPOUNDS TO AFLATOXIN BIOSYNTHESIS IN THE CORN INBRED LINE TEX6.

Zheng-Yu Huang[1], Donald G. White[2], and Gary A. Payne[1]. [1]North Carolina State University, Raleigh 27695-7616, and [2]University of Illinois, Urbana 61801.

The most effective control strategy for aflatoxin contamination is the development of host plant resistance. Identification and characterization of resistance to aflatoxin accumulation is laborious and expensive. The ability to rapidly screen for resistance to aflatoxin accumulation would greatly facilitate the identification of new sources of resistance and the subsequent movement of resistance genes into agronomically desirable lines. The objectives of our research was to: 1) to develop a reliable bioassay to evaluate corn lines for resistance to aflatoxin accumulation; 2) to identify resistant germplasm; and 3) to identify compounds in corn kernels responsible for resistance to aflatoxin contamination. The assay we have developed is based on the evaluation of corn seed extracts for the presence of compounds inhibitory to aflatoxin formation. To test the reliability of the assay, we examined corn genotype B73 whose derivatives are widely used commercially and considered susceptible to aflatoxin contamination, and a resistant line, Tex6, developed at the University of Illinois. Corn kernels were ground and an aqueous extract was added to a medium conducive for aflatoxin production. Aliquots of this extract were added to microtitre dishes and inoculated with conidia of *A. flavus* strain 13-22, a transformant that harbors a construct with the *ver1* promoter fused to the *E. coli uidA* gene (Flaherty et al., 1995). In our assay, the fungus produced significantly less aflatoxin and GUS activity on extracts of Tex6 (resistant) than extracts of B73 (susceptible). We also examined 9 other lines differing in resistance to aflatoxin accumulation in filed studies. In general, the ranking of these inbreds in the bioassay were similar to the ranking of the genotypes in field experiments. We have begun initial characterization of the inhibitory compound. The active compound is water soluble and heat labile at 100C; it retains 85% of its activity at 65C. The inhibitory compound is precipitated by $(NH_4)_2SO_4$ at 45-50% saturation. Analyses through DEAE anion exchange chromatography and native polyacrylamide gel electrophoresis suggests the presence of at least two compounds inhibitory to aflatoxin synthesis in the extract of Tex6. Both inhibit aflatoxin formation but one has greater inhibition of the fungal growth. The initial characterization indicates that one of the two compounds has a molecular weight greater than 15,000 daltons. The ability to identify compounds associated with resistance to aflatoxin accumulation in a laboratory assay such as the one we have developed would greatly facilitate breeding for resistance to aflatoxin accumulation, especially if the production of a specific compound(s) can be related to specific RFLP markers.

The Inhibition of Aflatoxin Production in *Aspergillus flavus* by Pistachio Hull

Noreen E. Mahoney and Anthony C. Waiss, Jr.
Western Regional Research Center, ARS, USDA, Albany, CA 94710

Wounded fresh pistachio hulls and kernels are readily colonized by *Aspergillus flavus*. High levels of aflatoxin are produced on wounded kernels; however, hulls completely suppress aflatoxin biosynthesis. Freeze-dried hulls were sequentially extracted with increasingly polar solvents and the extracts were tested for aflatoxin inhibitory activity. The water extract almost completely inhibited aflatoxin production in *A. flavus*. The water extract was further purified to remove sugars and inorganics, and this partially purified extract inhibited aflatoxin by about 50% at 0.02% (w/v) and 100% at 0.1% (w/v). Wounded fresh kernels were placed in a simulated flotation bath (used in pistachio processing) containing 1% (w/v) of this purified water extract and inoculated with *A. flavus*. These kernels developed 65% less aflatoxin and significantly less *A. flavus* sporulation than similarly wounded and inoculated kernels added to a flotation bath containing only water. Wounded fresh kernels kept in-hull and inoculated developed 96% less aflatoxin than the Wounded inoculated kernels floated in water. Kernels which had been contaminated with *A. flavus* prior to harvest could accumulate additional aflatoxin if irregularities in the drying or storage process leave kernels with enough moisture for fungal activity. The use of hull extract in the flotation bath could reduce additional aflatoxin accumulation in these kernels, and the reduction in sporulation could prevent the spread of *A. flavus* contamination to neighboring pistachios.

Aspergillus flavus Colonization and Aflatoxin Contamination in Wounded Pistachio Hulls and Kernels

	Kernels or Hulls with visible sporulation	Kernels or hulls with aflatoxin	Average Aflatoxin (µg/kernel)	Median Aflatoxin (µg/kernel)	Total Aflatoxin µg/50 kernels
In-hull					
Hulls	50/50	0/50	0	0	0
Kernels	NA*	38/50	1.8	0.6	92
Control bath					
Kernels	50/50	50/50	43	21	2150
Hull extract bath					
Kernels	29/50	46/50	15	3.5	750

*Not applicable

GENETIC MECHANISMS

Platform Poster Session- Fundamental Genetic Mechanisms Controlling Aflatoxin Biosynthesis:
D. Bhatnagar, F.S. Chu, N.P. Keller, J.E.Linz, G.A. Payne, C.P. Woloshuk

Many of the concepts discussed in this summary are presented in more detail in recently published articles by Bhatnagar et al. (Bhatnagar, D., T.E. Cleveland, G.A. Payne, and J.E. Linz. Mar., 1995. Molecular biology to eliminate aflatoxins. Inform 6:262-271) and Trail et al. (Trail., F., N. Mahanti, and J.E. Linz, 1995. Molecular biology of aflatoxin biosynthesis. Microbiol. 141:755-765). Reprints are available upon request.

Preharvest reduction or elimination of aflatoxin contamination of food or feed products can be accomplished at two levels: 1) block the interaction of the pathogen with the host plant; or 2) inhibit synthesis of aflatoxin by the pathogen. The biosynthesis group has generated tools which can be applied to aflatoxin elimination at both levels. For example, biocontrol strains of the fungus are available which can help prevent toxigenic fungi from interacting with the host plant. In addition, reporter constructs carried by "tester strains" are available which can be used to identify compounds (ie. natural plant products) which can inhibit fungal growth or aflatoxin biosynthesis. The research on aflatoxin biosynthesis has also resulted in very interesting observations which support a close association between aflatoxin biosynthesis and fungal development. The goal of this summary is to present research from the poster session at the 1995 Aflatoxin Elimination Workshop (AEW), "Fundamental genetic mechanisms controlling aflatoxin biosynthesis" which illustrates the tools which the biosynthesis group has made available to the other focus groups (crop resistance/genetic engineering/conventional breeding, microbial ecology, and crop management).

Before proceeding it is useful to emphasize several key points which lay the foundation for our strategy. 1) Aflatoxin synthesis is carried out by enzymes and regulatory proteins (trans-acting factors; TAF) encoded by many genes (16 or more). Cloning these genes and understanding their structure, function, and regulation was a primary emphasis of early work by the biosynthesis group. To this point most if not all of the genes have been localized to gene clusters in Aspergillus parasiticus, A. flavus, and A. nidulans (Brown et al., 1995; Yu et al., 1995) and the function of many of these genes has been assigned using a variety of techniques including nucleotide sequence, gene disruption, Western blot, and enzyme activity analyses as well as metabolite conversion studies, protein purification, and generation of antibodies to native proteins or proteins synthesized in Escherichia coli. This information was summarized in posters by Trail et al. (Linz, MSU) and Brown et al. (Keller; Texas AM Univ.). **These genes are essential tools which are now being used to identify possible targets available for inhibiton of fungal growth and aflatoxin synthesis.** 2) Gene expression is a multistep process which includes transcription, RNA processing and transport, translation, posttranslational processing and localization. **If you block expression of a gene at any step you prevent its function.** For example, preventing the synthesis or binding of TAF to gene promoters will prevent their transcription. Similarly, polyclonal antibodies, specific chemical compounds, or limitation for substrates or enzyme cofactors can be utilized to inhibit enzyme acitivity and/or protein localization. Clearly, blocking expression of genes involved in early steps in AFB1 synthesis would be most useful because you prevent accumulation of potentially toxic pathway intermediates. However understanding the function and regulation of genes later in the pathway is necessary to provide a complete picture of the potential importance of this pathway to the producing organism (see below). **3) In order to effectively prevent aflatoxin contamination,**

an attack at multiple levels is the best approach. For example, if you reduce interaction of the pathogen with the plant by 80%, and then block expression of one or more early genes in the pathway by 80%, this combination results in a huge impact on the level of contamination (96% reduction) of plant-derived products. **This also is more likely to reduce problems associated with microbial resistance because the pathogen must adapt to several layers of protection simultaneously. 4) Part of this multi-level approach entails identifying and enhancing the natural resistance mechanisms of the host plant as well as the utilization of antifungal genes from microbial or plant sources.**

Three key tools generated by the biosynthesis group will be reviewed: 1) biocontrol strains; 2) tester strains; 3) purified native proteins, protein synthesized in E. coli, and antibodies raised to these proteins. In addition, basic research in the areas of gene regulation and fungal development will be reviewed with special emphasis on the application of this knowledge to preharvest aflatoxin elimination.

Biocontrol strains- Over the past few years, Peter Cotty and Richard Cole have reported promising results with the use of fungal strains to reduce toxin levels on cotton and peanuts. Fortunately, field isolates of aflatoxin non-producing strains of Aspergillus flavus were provided to us by Nature. However to utilize Aspergillus parasiticus in analogous biocompetitive experiments in peanut soils, it has been necessary to generate aflatoxin non-producing mutants which are blocked early in the aflatoxin biosynthetic pathway, since there are no "natural" strains of Aspergillus parasiticus that do not accumulate aflatoxin or other harmful "end pathway" intermediates (eg. 0-methylsterigmastocystin). Since most fungal mutagenizing agents are random in their effects and could effect genes involved in the biocompetitiveness of potentially useful strain, a more targeted approach to specifically disrupt aflatoxin biosynthetic genes is desirable. Genes encoding activities from early in AFB1 synthesis have been identified for use in gene disruption studies, including those encoding putative polyketide synthetase (PKS) and fatty acid synthetase (FAS) activities. Trail et al. (Linz, AEW poster) report that these genes have been specifically disrupted in A parasiticus resulting in strains which do not synthesize any detectable AFB1 or other known pathway intermediates (Chang et al, 1995; Trail et al., 1995; Mahanti et al., 1996). These strains are available for testing for effectiveness in greenhouse or field trials. Yu et al. (Bhatnagar, AEW poster) report that the function of another early gene, avnA has been assigned using gene disruption demonstrating that this approach is a powerful tool to simultaneously study gene function and to generate "safe" biocontrol strains.

Tester strains- Gary Payne (NC State) and Charlie Woloshuk (Purdue) generated strains of A. flavus containing a reporter gene (GUS) fused to the ver-1 promoter (a gene involved in aflatoxin biosynthesis) and, using a simple fluorometric assay, showed that expression of GUS protein activity followed the same time course as aflatoxin synthesis (Flaherty et al., 1995). At this workshop, both scientists demonstrated the utility of these strains in identifying plant compounds which induce or inhibit aflatoxin synthesis at the transcriptional level. Huang et al. (Payne, AEW poster) report the presence of compounds in seed extracts of Tex6, a line of corn with resistance to aflatoxin accumulation developed at the University of Illinois by Donald White. Preliminary characterization of the extracts indicate that two compounds are present, one that inhibits fungal growth and and aflatoxin, and one that inhibits aflatoxin synthesis but not fungal growth. These results are exciting because for the first time it may be possible to identify one mechanism of resistance to aflatoxin accumulation. Woloshuk (AEW poster) reports that extracts of corn kernels colonized with a toxigenic strain of A. flavus could induce aflatoxin synthesis and GUS expression in A. flavus containing the ver-1/GUS reporter construct. The inducing activity was a heat stable, low molecular weight compound which was characterized to contain the sugars glucose, maltose, and maltotriose. Since these molecules were apparent breakdown products of starch and induced aflatoxin synthesis in a dose dependent fashion, it was hypothesized that fungal amylase generated the sugars which in turn induced aflatoxin gene expression. Amylase activity was detected in the kernel extracts in support of this theory. One potential application of this

knowledge is that inhibitors of fungal amylase may inhibit aflatoxin induction in infected kernels providng a direct way to attack the fungus growing on the host plant.

Proteins/antibodies- Polyclonal or monoclonal antibodies generated against enzymes or regulatory proteins involved in aflatoxin biosynthesis can serve many functions. The antibodies can be used directly to purify large quantities of the native proteins from the fungus or to clone the relevant genes. With pure proteins, studies focused on determining enzyme activity and inhibitors of enzyme function are possible. The antibodies can also be used to study patterns of protein expression and/or protein localization. These in turn provide potential targets to block aflatoxin synthesis. F.S. Chu (Univ. Wisconsin, AEW poster) reports the successful production of antibodies to several proteins involved in AFB1 synthesis including the gene products of nor-1, ver-1, pksA (enzymes), and aflR (TAF). He also reports the production of anti-NSR (norsolorinic acid reductase; product of nor-1) in Escherichia coli, providing a rapid mechanism to generate an essentially unlimited supply of these antibodies in a prokaryotic cell. Chu (AEW poster) and Trail et al. (Linz, AEW poster) demonstrate the utility of these antibodies in analysis of aflatoxin gene expression at the level of protein synthesis and accumulation.

Regulation of AFB1 gene expression- Although studies on understanding AFB1 gene promoter structure and function are focused on basic biological mechanisms, these studies have the potential to generate important breakthroughs in aflatoxin elimination. For example, identification of TAF (like aflR) regulating AFB1 gene expression provides targets for gene inactivation. Compounds which inhibit TAF binding or synthesis should eliminate AFB1 gene expression at the level of transcription. Meyers et al. (Payne, AEW poster) and Trail et al. (Linz. AEW poster) report progress in studies on the promoters for aflR, aflJ, and nor-1. Both groups have identified several putative sites for TAF/promoter interactions. The identity of these sites needs to be experimentally confirmed. Chang et al. (Bhatnagar, AEW poster) report progress on understanding nitrate repression of aflatoxin gene function through their studies on the niaD and niiA intergenic promoter region.

Cell development- For many years, a suspected connection between aflatoxin synthesis and fungal development has been reported (Bennett et al., 1986; Cotty, 1988). Studies of UV or gene disruption mutant strains of A. parasiticus, which accumulate different aflatoxin pathway intermediates, suggest that these pathway intermediates may downregulate sclerotia development (Skory et al., 1992; Trail et al., 1995; Mahanti et al., 1996). Bhatnagar et al. (AEW poster) report isolation of variants of A. parasiticus upon serial transfer. These isolates, called Sec (-), were defective in conidiation and aflatoxin synthesis. The aflatoxin genes were not deleted in Sec (-) strains however accummulation of specific AFB1 transcripts (nor-1, omt-1, and aflR) did not occur. The data suggest that regulation of AFB1 and conidiation are linked. In a related observation, Nancy Keller reported that flbA mutants of A. nidulans are simultneously defective in conidiation and sterigmatocystin synthesis. Complementation of flbA restores both phenotypes to wild type. Perhaps, alteration in the function of flbA or a similar gene is responsible for the phenotype of Sec (-) variants. Together these data suggest strongly that secondary metabolism and conidiation (fungal development) are subject to global regulation. This may in turn suggest that strategies may be developed which can inhibit toxin synthesis and the ability of the fungus to disperse or survive in the environment.

References

Bennett, J.W., P-M. Leong, S. Kruger, and D. Keyes. 1986. Sclerotial and low aflatoxigenic morphological variants from haploid and diploid Aspergillus parasiticus. Experientia

42:848-851.

Brown, D.W., J-H Yu, H.S. Kelkar, M. Fernandes, T.C. Nesbitt, N.P. Keller, T.H. Adams, and T.J. Leonard. Twenty-five co-regulated transcripts define a sterigmatosystin gene cluster in Aspergillus nidulans. 1995. USDA-ARS Aflatoxin Elimination Workshop, Atlanta GA.

Chang, P-K., J.W. Cary, J-J. Yu, D. Bhatnagar, and T.E. Cleveland. Aspergillus parasiticus pksA, a homolog of Aspergillus nidulans *wA*, is required for aflatoxin B1biosynthesis. 1995. Mol. Gen. Genetic. (In press).

Cotty, P.J. 1988. Aflatoxin and sclerotial production by Aspergillus flavus. Phytopathology 78:1250-1253.

Flaherty, J.E., M.A. Weaver, G.A. Payne, and C.P. Woloshuk. 1995. A beta-glucuronidase reporter gene construct for monitoring aflatoxin biosynthesis in Aspergillus flavus. Appl. Env. Microbiol. 61:2482-2486.

Mahanti, N., D. Bhatnagr, J.W. Cary, and J.E. Linz. 1996. Structure and function of fas-1A, a putative fatty acid synthetase directly involved in aflatoxin biosynthesis in Aspergillus parasiticus. Appl. Env. Microbiol. 62 (In press).

Skory, C.D., P-K. Chang, J.W. Cary, and J.E.Linz. 1992. Isolation and characterization of a gene from Aspergillus parasiticus associated with conversion of versicolorinA to sterigamatocystin in aflatoxin biosynthesis. Appl. Env. Microbiol. 58:3527-3537.

Trail, F., N. Mahanti, M. Rarick, R. Mehigh, S-H. Liang, R. Zhou, and J.E. Linz. 1995. A physical and trancriptional map of an aflatoxin gene cluster in Aspergillus parasticus and the functional disruption of a gene involved early in the aflatoxin pathway. Appl. Env. Microbiol. 61:2665-2673.

Yu, J-J., P-K. Chang, J.W. Cary, M. Wright, D. Bhatnagar, T.E. Cleveland, G.A. Payne, and J.E. Linz. 1995. Comparative mapping of aflatoxin pathway gene clusters in Aspergillus parasiticus and Aspergillus flavus. Appl. Env. Microbiol. 61:2365-2371.

IMMUNOCHEMICAL STUDIES OF THE ENZYMES OF AFLATOXIN BIOSYNTHESIS

F. S. Chu[a], R. C. Lee[a], B. H. Liu[a], J. Cary[b], D. Bhatnagar[b] & T. E. Cleveland[b]

[a] Food Research Institute, University of Wisconsin-Madison, WI, and
[b] Southern Regional Research Center, USDA, New Orleans, LA

Attempts were made to produce both monoclonal (Mab) and polyclonal (Pab) antibodies against several key enzymes and regulatory proteins involved in aflatoxin (AF) biosynthesis, i.e. sterigmatocystin (ST) methyltransferase (ST-MT), norsolorinic acid reductase (NSR), Ver-1, polyketide synthase (PKS) and *afl-R* protein. In the last few years, we have sucessfully obtained both Mab and Pab for NSR and ST-MT. Effective enzyme-linked immunosorbent assays (ELISA) were established for these enzymes and subsquently used in a study elucidating the role of these enzymes in AFB1 formation. During 1994-95, much of our effort was devoted to the production and characterization of antibodies against Ver-1, PKS, and *afl-R* protein.

A. Research Progress:

1. Production and characterization of antibodies against PKS: Polyclonal antibodies against polyketide synthase (PKS), the enzyme involved in the initial step of AF biosynthesis, were produced in rabbits. Two immunogens were prepared by conjugating two selected peptide fragments, one with amino acid (a.a.) sequence identical to the partial fragment to both patulin and AFB PKS and the other with a.a. sequence identical to part of the fragment of only AFB PKS, to a branch-mutiple lysine chain, respectively. Antibodies showed good specificity and affinity toward the respective immunogens. Western blot analysis revealed that the antibodies reacted with a specific protein band, corresponding to 200-220 kD in SDS-PAGE, in the crude protein preparations from *Aspergillus flavus* strain NRRL 3357 grown in aflatoxin-supportive medium. The specific band was absent in the extracts from the fungus grown in the nonsupportive medium. Such data indicate that the antibodies are capable of identifying the PKS in the fungus extract. Thus, we plan to characterize both the antibodies and the enzymes through: (i) purification of PKS from AFB producing fungi and from *E. coli* that PKS gene was expressed (Chang et al., *Mol. Gen. Genet*, **1995**, 248: 270) by immunoaffinity method; and (ii) comparing the kinetics of AFB production and the formation of PKS by ELISA and Western blot analyses for the AFB producers and non-aflatoxin producers.

2. Production and characterization of antibodies against AFLR protein: Both Pab and Mab against AFLR1 protein, the gene product of *afl-R*, were generated after immunizing rabbits and mice with a fragment of recombinant AFLR1 (amino acid residues 85-169) expressed in *E. coli*. Western blot analysis and ELISA revealed that both types of antibodies recognize the recombinant AFLR1 protein from *A. parasiticus* and the recombinant AFLR2 protein from *A. flavus*. However, no specific band corresponding to AF formation was detected in the crude extracts or the extracts from the nuclei preparation. Two new immunogens were prepared and immunized to the rabbits for antibody production. The immunogens were prepared by conjugation of two peptide fragement from the deduced a. a. sequence of the AFLR protein, one from residues 29 to 52 (zinc finger area) and the other from residue 124 to141, via the multiple antigen chain method, as those for the PKS. Preliminary ELISA data showed that Pab against recombinant AFLR1 also recognize these two immunogens.

3. Production and characterization of antibodies against ver-1: Antibodies against ver-1, a protein/enzyme involved in the biosynthesis of AFB, were purified from a batch of antiserum raised against a semi-purified NSR extract from *A. parasiticus* SRRC 2043. An affinity column in which the

purified ver-1 was conjugated to the sepharose gel was used in the purification step. Immunoblotting analysis of the purified ver-1 protein and various fungal extracts revealed that the purified antisera not only were highly specific to the ver-1 but also cross-reacted with ver-A protein from *A. nidulans*. An indirect ELISA was developed for monitoring the ver-1 protein. All AFB and ST producers examined contained the ver-1. Kinetic analysis of the appearance of the ver-1 protein by various fungi with ELISA revealed that the accumulation of ver-1 protein was 24 to 48 hours prior to AF formation. The antibody was also used at SRRC to clone the ver-1 gene from a cDNA library; the gene that was cloned earlier in Dr. John Linz's lab by genetic complementation of the ver-1 from *A. parasiticus*.

4. Kinetic study of the correlation between the formation of NSR and ver-1 protein formation and the production of AFB1: *Aspergillus parasiticus* NRRL 13007, NRRL 2999, *A. flavus* NRRL 3357 and NRRL 5565 were grown under aflatoxin-inducing conditions. *A. parasiticus* NRRL 13007 was also inoculated in peptone mineral media (PMS) that does not support AFB formation. AFB was detected in *A. parasiticus* NRRL 13007, NRRL 2999 and *A. flavus* NRRL 3357, but not in the other two cultures. We found high levels of NSR and ver-1 in the AFB producers; NSR was produced before ver-1 protein and AFB was produced last. Small amounts of NSR and ver-1 were detected in the AFB non-producers, probably due to the non-specific binding of the antibodies. Further purification of the antibodies through a column armed with culture extract from *A. parasiticus* NRRL 13007 grown in PMS decreased the non-specific binding of antibodies. In the Western blot, protein bands of NSR and ver-1 were observed only in the AF producers.

5. Cloning the gene of anti-NSR antibodies from hybridoma cells to E. coli: The gene fragments of light and heavy chain variable regions for anti-NSR Mab were linked together (ScFv) and ligated into pCANTAB 5 E vector. The ScFv fragment of the antibodies was expressed in *E. coli* TG-1 as fusions with phage protein and displayed on the phage surface. Soluble antibodies can be produced after infection of *E. coli* HB2151 with antigen-positive phage clone. The antibodies were extracted from the *E. coli* cells. The sensitivity of the antibody was very similar to the original antibodies produced by hybridoma cells. However, the titer was low.

B. Implication of research data: Over the years, we have collaborated with scientists at USDA and several universities (U. of Wisconsin, North Carolina State, Michigan State and Texas A & M) by supplying appropriate immunochemical reagents. Antibodies have played an important role for the success in coloning the genes for several key enzymes for AFB biosynthesis. With the availability of antibodies, specific ELISA methods for these enzymes have been established. These methods have proved to be very effective in diffierentiating AFB producers from non-producers. They could be used as a complimentary method to detect the possible presence of AFB in the samples. These antibodies have been used in immunoaffinity chromatography as a simple method for purification of these enzymes. All these the technologies could be transferred to other scientists to facilitate further work on the control of aflatoxins in field crops.

C. Publications:

1. Yu, J., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Keller, N.P. and Chu, F. S. 1993. Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an O-methyltransferase involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 59: 3564-3571.
2. Liu, B. H., Keller, N. P., Bhatnagar, D., Cleveland, T. E. and Chu, F. S. 1993. Production and characterization of antibodies against sterigmatocystin O-methyltransferase. Food & Agric. Immun. 5:155-164.
3. Lee, R. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E. and Chu, F. S. 1995. Production and characterization of antibodies against norsolorinic acid reductase. Food & Agric. Immun.7:21-32.

Is Regulation of Aflatoxin Biosynthesis Correlated with Fungal Development?

Deepak Bhatnagar[1], Shubha P. Kale[2], Jeffrey W. Cary[1], Perng-Kuang Chang[3], J. W. Bennett[3] and Thomas E. Cleveland[1] [1] USDA, ARS, SRRC, New Orleans, LA; [2] Xavier University, New Orleans, LA; and [3] Tulane University, New Orleans, LA.

Aflatoxin synthesis in *Aspergillus parasiticus* and *A. flavus* is considered to be a "secondary" metabolic process and has no obvious physiological role in primary growth and metabolism of the organism. As yet, there is no confirmed biological role of aflatoxin in the ecological survival of the fungal organisms. Because aflatoxins are toxic to certain potential competitor microbes in the ecosystem and insect pests of crops inhabitated by the *aspergilli*, a survival benefit to toxigenic fungi is implied. It should be noted, however, that aflatoxin *per se* is a poor antibiotic.

However, it has been recently determined that aflatoxin synthesis occurs in a very complex and highly organized manner. The genes involved are organized systematically in a cluster on one of the fungal chromosomes, suggesting that aflatoxins may have some significant function in the life cycle or survival of the fungus. Conidia (asexual spores) and sclerotia (resting/survival structures) are specialized structures used by the fungus for dissemination and survival, respectively. Recent observations from our lab and those from J. Linz's lab have suggested that normal sclerotia development may be correlated to normal aflatoxin production.

In the study presented here we demonstrated that alterations in the regulation of aflatoxin biosynthesis are correlated with alterations in the conidial morphology of the fungus. Six non-aflatoxigenic variants of *A. parasiticus*, isolated after 5 to 12 serial transfers of non-sporulating mycelia and designated *sec-*, were characterized morphologically by electron microscopy, biochemically by biotransformation studies with aflatoxin precursors and genetically by Northern hybridization analysis of aflatoxin biosynthetic gene transcripts. Scanning electron micrographs demonstrated that, compared to the parental *sec+* forms, the variant *sec-* forms had an abundance of vegetative mycelia, a significantly reduced number of conidiophores and conidia, and the presence of abnormal metullae. All *sec+* forms but none of the *sec-* forms showed bioconversion of sterigmatocystin (ST) to aflatoxins. Northern blots probed with pathway genes demonstrated lack of expression of both the aflatoxin biosynthetic pathway structural (*nor-1, omt-1*) and regulatory (*aflR*) genes in the *sec-* forms; PCR analysis confirmed the presence of the genes in the *sec-* genomes. These results indicate that the loss of aflatoxigenic capabilities in the *sec-* form may be the result of some regulatory abnormality, which is also associated with abnormal fungal morphology. Therefore, the possibility exists that the regulations of aflatoxin synthesis and fungal development may be interlinked.

PUBLICATIONS (since 1994 Workshop)

1. D. Bhatnagar, G. A. Payne, J. E. Linz and T. E. Cleveland. "Molecular Biology to Eliminate Aflatoxins." INFORM Vol. 6 pp. 262-271. (1995)

2. J. Yu, P-K Chang, J. Cary, M. Wright, D. Bhatnagar, T. E. Cleveland, G. A. Payne and J. E. Linz. "Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*." Appl. Environ. Microbiol. 61:2365-2371 (1995)

3. M. Wheeler and D. Bhatnagar. "Inhibition of aflatoxin production by *Aspergillus flavus* with pentachlorobenzyl alcohol, fthalide and pyroquilon." Pesticide Biochemistry and Physiology. 59:109-115. (1995)

4. P-K. Chang, J. W. Cary, J. Yu, D. Bhatnagar and T. E. Cleveland. "*Aspergillus parasiticus* polyketide synthase gene pksA, a homolog of *Aspergillus nidulans* wA, is required for aflatoxin B_1 biosynthesis." Mol and Gen Genet. 248:270-277. (1995)

5. C. P. Woloshuk, G. L. Yousibova, J. A. Rollins, D. Bhatnagar, and G. A. Payne. "Molecular characterization of the afl-1 locus in *Aspergillus flavus*." Appl. Environ. Microbiol. 61:3019-3023. (1995)

6. P-K. Chang, K. C. Ehrlich, J. Yu, D. Bhatnagar and T. E. Cleveland. "Increased expression of *A. parasiticus* aflR encoding sequence-specific DNA binding protein relieves nitrate inhibition of aflatoxin biosynthesis." Appl. Environ. Microbiol. 61:2372-2377. (1995)

7. N. P. Keller, S. Segner, D. Bhatnagar, and T. H. Adams. "stcS encodes a putative P450 monooxygenase needed for the conversion of versicolorin A to sterigmatocystin in *A. nidulans*." Appl. Environ. Microbiol. 61:3628-3632. (1995)

8. J. W. Cary and D. Bhatnagar. "Nucleotide sequence of an *Aspergillus parasiticus* gene strongly repressed by thiamine." Biochem. Biophys. Acta 1261:319-320. (1995)

9. P-K. Chang, D. Bhatnagar, T. E. Cleveland and J. W. Bennett. "Sequence variability in homologs of the aflatoxin pathway gene *aflR* distinguishes species in *Aspergillus* section *Flavi*. Appl. Environ. Microbiol. 61:40-43. (1995)

10. R. C. Lee, J. W. Cary, D. Bhatnagar and F. S. Chu. "Production and characterization of polyclonal antibodies against norsolorinic acid reductase

involved in aflatoxin biosynthesis. Food and Agricultural Immunology. 7:21-32. (1995)

11. N. P. Keller, D. Brown, R.A.E. Butchko, M. Fernandes, H. Kelkar, C. Nesbitt, S. Segner, D. Bhatnagar, T. E. Cleveland and T. H. Adams. A conserved polyketide mycotoxin gene cluster in *Aspergillus nidulans*. In Molecular Approaches to Food Safety Issues Involving Toxic Microorganisms. J. L. Richard, ed. (in press) (1995)

12. G. A. Payne, D. Bhatnagar, T. E. Cleveland and J. E. Linz. "Genomic organization and regulation of aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus*." In Molecular Approaches to Food Safety Issues Involving Toxic Microorganisms. J. L. Richard (ed). (in press) (1995)

13. M. A. Klich, J. Yu, P-K Chang, E. J. Mullaney, N. P. Keller, D. Bhatnagar and T. E. Cleveland. "Homology of genes involved in aflatoxin biosynthesis to DNA of aflatoxigenic and non-aflatoxigenic Aspergilli." Current Genetics (in press) (1995)

14. J. Yu, P. K. Chang, G. A. Payne, J. W. Cary, D. Bhatnagar and T. E. Cleveland. "Cloning and comparison of omt-1 gene encoding an O-methyltransferase involved in aflatoxin biosynthesis from *A. parasiticus* and *A. flavus*." Gene (in press) (1995)

15. J. W. Cary, M. S. Wright, D. Bhatnagar, L. Rachel and F. S. Chu. "Regulation of the *Aspergillus parasiticus* alcohol dehydrogenase gene ADH-2, is analogous to that of the aflatoxin biosynthetic gene, VER-1." Appl. Environ. Microbiol. (submitted)

16. S. P. Kale, J. W. Cary, D. Bhatnagar and J. W. Bennett. "Characterization of experimentally-induced, nonaflatoxigenic variant strains of *Aspergillus parasiticus*." Appl. Environ. Microbiol. (submitted)

CHARACTERIZATION OF THE AFLATOXIN BIOSYNTHETIC GENE *aflJ* AND THE INTERGENIC REGION BETWEEN *aflJ* AND THE REGULATORY GENE, *aflR*. D. M. Meyers, K. R. Foutz, and G. A. Payne. Department of Plant Pathology, North Carolina State University, Raleigh 27695-7616.

Aflatoxin biosynthesis is regulated by a pathway regulatory gene *aflR*. Adjacent to *aflR* in the biosynthetic cluster of *Aspergillus flavus* is a gene of unknown function. The transcription of this gene, designated *aflJ*, is directly correlated with aflatoxin biosynthesis. We have shown by gene disruption that *aflJ* is required for aflatoxin biosynthesis. A disruption vector was constructed by inserting the *pyr-4* gene into the coding region of *aflJ*. Transformation of this disrupted copy of *aflJ* into an aflatoxin-producing *pyrG* strain of *A. flavus* resulted in the displacement of the wild-type copy of *aflJ*. These disrupted transformants lacked the ability to produce aflatoxin; aflatoxin production could be restored by complementation of the disrupted strain with a wild-type copy of *aflJ*. Partial sequence analysis of the gene has revealed the presence of two introns and three exons. A major open reading frame has been identified which shows no known homology to any known proteins in the databases. The *aflJ* gene is of particular interest because of its close proximity to *aflR*. The two genes are divergently transcribed and their translational start sites are only 737 bp apart. We are testing the hypothesis that these two genes share promoter activities and are coordinately regulated. In order to study the promoter activity of the intergenic region between these two genes, we have fused the 737 promoter region in both orientations to a GUS reporter gene, and have shown that GUS expression by both the *aflR*::GUS and the *aflJ*::GUS constructs follows aflatoxin accumulation. Deletions in the promoter regions in both orientations are being examined to determine the functional regions in the promoter and to determine if the two genes share functional regions.

Controlling Aflatoxin Production in Maize Through the Identification of Inducing Metabolites

Charles P. Woloshuk, Deptartment of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907

The goal of this research is to identify metabolites in maize kernels that induce aflatoxin biosynthesis in *Aspergillus flavus*. These metabolites could be considered susceptibility factors that are responsible for high levels of aflatoxin production in certain maize lines. The rationale for pursuing this research is the potential of finding new strategies for controlling aflatoxin contamination. Potential targets include inhibiting the production of these inducing metabolites or interferring with their utilization by the fungus.

We have developed a method to study the induction and regulation of aflatoxin biosynthesis by examining the expression of one aflatoxin pathway gene, *verI*. The promoter region of *verI* was fused to the ß-glucuronidase (GUS) gene (*uidA*) from *E. coli* to form the reporter construct, GAP13. *A. flavus* strain 656-2 was transformed with this construct. Aflatoxin production, GUS activity, and transcript accumulation were determined in transformants after shifting the cultures from a medium non conducive to a medium conducive for aflatoxin biosynthesis. Transformants harboring GAP13 displayed GUS expression only when aflatoxin was detected in culture. Further, the transcription of the *uidA* gene driven by the *verI* promoter followed the same profile as for the *verI* genes (Flaherty, J. E., M. A. Weaver, G. A. Payne, and C. P. Woloshuk. 1995. Appl. Environ. Microbiol. 61:2482-2486).

Using the GUS-expressing transformant in a bioassay, an aflatoxin-inducing activity was detected in extracts (EF) from ground maize kernels colonized with the aflatoxigenic strain NRRL 3357. The inducing component was water soluble and was not inactivated by autoclaving for 15 minutes. The extract was passed though ultrafiltration membranes with 100- and 10-kDa exclusion. Analysis of the filtrates indicated that the inducing activity in the extract passed through the filter.

Amylase activity was detected in the extract. The presence of amylase and the small molecular size of the inducing component suggested that the inducing activity may be the products starch degradation (glucose, maltose and maltotriose). The aflatoxin-inducing activity of these sugar molecules has been well documented in the literature. Total sugar (glucose equivalents) in the EF-extract was 295 mM and in the filtrate passing through the 10-kDa filter EF-10 was 202 mM. HPLC analysis of EF and EF-10 indicated the presence of glucose, maltose, and maltotriose in near equal molar concentrations (about 1.5 mM). These products suggest that the starch degradation enzyme is an α-amylase in the extracts. When the extracts were analyzed by isoelectric-focussing gel electrophoresis, the amylase activity migrated as a single band with a pI of 4.4.

To determine how the concentration of the starch degradation products affect the induction of aflatoxin as measured by transcription from the *verI* promoter, various concentrations of glucose, maltose and maltotriose were tested in the bioassay. For all three sugar molecules, there was a positive correlation between the level of GUS activity induced in the bioassay and the sugar concentration. The minimum concentrations that induced measurable GUS activity were 2.8 mM glucose, x mM maltose, and 1 mM maltotriose. These data indicate that induction of aflatoxin by these sugars is not triggered by a threshold concentration. Rather, it is possible that induction process is modulated by the concentration of sugar.

There are several reports indicating that upon infection of maize kernels by *A. flavus* the fungus preferentially colonizes the embryo tissue. All indications are that the endosperm tissue is not significantly affected by the fungus until late in the destruction of the kernel. Aflatoxin is also produced predominately in the embryo tissue. *Aspergillus flavus* may colonize the embryo tissue first because it is richer than the endosperm in nitrogen and other nutrients. The data we have obtained thus far supports the hypothesis that after the colonization of the embryo, *A. flavus* produces a extracellular amylase that supplies a burst of fermentable sugars, resulting in the induction of aflatoxin. Starch is the primary component of the endosperm; however, the embryo also has significant amounts of starch; as much as 8% of the dry weight.

Title: Twenty-five Co-regulated Transcripts Define a Sterigmatocystin Gene Cluster in *Aspergillus nidulans*

Brown, D.W.*, Yu, J.-H.‡, Kelkar, H.S.¶, Fernandes, M.¶, Nesbitt, T.C.*, Keller, N.P.*, Adams, T.H.¶, and Leonard, T. J.‡

* Department of Plant Pathology and Microbiology
¶ Department of Biology
Texas A&M University
College Station, TX 77843

‡ Department of Genetics
University of Wisconsin
Madison, WI 53706

Abstract. Sterigmatocystin (ST) and the related fungal secondary metabolites, aflatoxins (AFs), are among the most toxic, mutagenic, and carcinogenic natural products known. The ST biosynthetic pathway in *Aspergillus nidulans* is estimated to involve at least 15 distinct enzymatic activities, while certain *A. parasiticus*, *A. flavus*, and *A. nomius* strains contain additional activities that convert ST to AF. We have characterized a 60 kb region in the *A. nidulans* genome and find it contains many, if not all, of the genes needed for ST biosynthesis. This region includes *verA*, a structural gene previously shown to be required for ST biosynthesis, as well as 24 additional, closely-spaced transcripts ranging in size from 0.6 to 7.2 kb, that are coordinately induced only under ST-producing conditions. Each end of this gene cluster is demarcated by transcripts that are expressed under both ST-inducing and non-ST-inducing conditions. Deduced polypeptide sequences of regions within this cluster had a high percentage of identity with enzymes having activities predicted for ST/AF biosynthesis, including a polyketide synthase, a fatty acid synthase (α and β subunits), five monooxygenases, four dehydrogenases, an esterase, an *O*-methyltransferase, a reductase, an oxidase, and a zinc cluster DNA binding protein. A revised system for naming the genes of the ST pathway is presented.

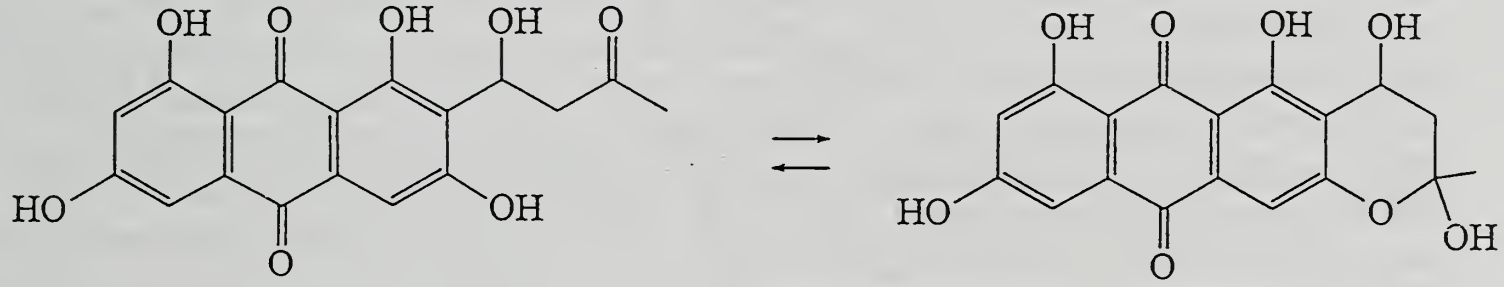
A NEW METABOLITE OF *ASPERGILLUS PARASITICUS* AS A POSSIBLE BIOSYNTHETIC PRECURSOR OF AFLATOXINS

V.S. Sobolev, R.J. Cole, J.W. Dorner and B.W. Horn
National Peanut Research Laboratory, USDA, ARS, 1011 Forrester Dr., Dawson, GA 31742
G.G. Harrigan and J.B. Gloer
The University of Iowa, 305 Chemistry Botany Blvd., Iowa City, Iowa 52242

Aflatoxins are carcinogenic secondary metabolites of *Aspergillus flavus* and *A. parasiticus*. Biochemical and genetic studies of aflatoxin biosynthesis have resulted in a better understanding of the genes, enzymes and precursors involved in the pathway. However, not all of the molecular transformations within the pathway have been determined. The isolation and structural elucidation of new aflatoxin intermediates is, therefore, critical.

The purpose of this work was to isolate a new colored compound produced by a mutant strain of *A. parasiticus*, to determine its structure, and to estimate its role as a possible biosynthetic precursor of aflatoxins.

An *A. parasiticus* mutant (ATCC 20979) was obtained through UV-irradiation of a wild-type isolate (ATCC 62882) that produced *O*-methylsterigmatocystin. After incubation of the mutant strain on a liquid medium, the orange pigment was extracted with chloroform. Several chromatographic clean-up procedures (silica gel) and crystallizations resulted in the isolation of an orange crystalline compound (temporarily called U_1). The compound possessed chemical and spectral properties similar to those of the aflatoxin precursors versicolorins. The structural elucidation of U_1 using NMR (^{13}C), PMR (^{1}H), IR, UV and mass spectrometry resulted in the structure shown in Fig. 1a ($C_{18}H_{14}O_8$, MW 358).



a **b**

Fig. 1

In polar solvents the pigment exists as an equilibrium mixture of isomers (Fig. 1 a and b).

To determine the possible biosynthesis of the new metabolite U_1 by wild-type isolates of *A. parasiticus*, an HPLC method for the simultaneous detection and quantitation of U_1 and versicolorins in synthetic medium was developed. The extraction and clean-up procedure included water-chloroform partition at basic/acidic pH. HPLC was performed on silica gel using hexane - 2-propanol - water - acetic acid (1100+300+20+3 v/v's) as a mobile phase.

Preliminary studies showed that some wild-type isolates (both aflatoxin producers and nonproducers) of *A. parasiticus* produced U_1. The initial wild-type isolate of *A. parasiticus* (ATCC 62882) did not produce U_1 under the same conditions. The *A. parasiticus* mutant (ATCC 20979) produced versicolorin A (Ver A) as the only metabolite after 3 days of incubation (30°C). At 4 days the concentration of Ver A remained almost unchanged and U_1 pigment was first detected, while after 5 days Ver A disappeared and U_1 was the major metabolite.

The significance of U_1 as a possible intermediate in the aflatoxin biosynthetic pathway is based on its chemical structure (anthraquinone moiety, typical to biosynthetic precursors of the aflatoxins and sterigmatocystins) as well as its production by wild-type isolates of *A. parasiticus*.

Further biochemical work is necessary to confirm its role in the biosynthesis of aflatoxins.

Molecular approaches to preharvest elimination of aflatoxin contamination

F. Trail, N. Mahanti, L. Roze, T-S Wu, S-H Liang, R. Zhou, M. Rarick, M. Miller, and J.E. Linz
Dept. Food Science and Human Nutrition, Michigan State University, E. Lansing, MI 48824

Work in our lab has focused on understanding the structure, function, and regulation of expression of five genes involved in aflatoxin B1 (AFB1) biosynthesis in Aspergillus parasiticus. The genes nor-1, ver-1A, fas-1A, fas-2A, and pksA, are clustered in a 65-kb region in one chromosome of A. parasiticus. Utilization of methods including nucleotide sequence, gene disruption, and western blot analyses, together with enzyme activity assays, and metabolite conversion studies have allowed the assignment of specific functions for each of the 5 genes in the AFB1 biosynthetic pathway. The goals of this poster were: 1) to present examples of the methods used in analysis of AFB1 gene structure/function; and, 2) to highlight tools which have been generated by this work- these tools are available to be used to help reduce or eliminate AFB1 contamination of plant or animal-derived products and to increase our basic understanding of the biology of AFB1 synthesis.

The primary tools which have been developed as a result of work in our lab and the labs of the other members of the biosynthesis group are: 1) **biocontrol strains** (pksA, fas-1A, avnA, nor-1, ver-1A gene knockout strains; Chang et al., 1995; Trail et al., 1995; Mahanti et al., 1995; 1995 Workshop Posters, Trail, Yu)- these strains may be useful in excluding toxigenic fungi from infecting the host plant; 2) **fungal tester strains** containing GUS reporter constructs (Trail and Linz, 1994; Flaherty et al., 1995) or pathway blocked mutants (Nancy Keller, Texas A&M)- these strains may be used to measure host plant resistance to fungal growth or toxin production as well as to investigate the effect of natural plant products on AFB1 gene expression to identify inducers or inhibitors (1995 Workshop Posters: Woloshuk, Purdue; Payne, NC State); and 3) **PAb raised to pure native proteins or proteins expressed in E. coli** (Liang and Linz 1994; 1995 Workshop Posters: FS Chu, U. Wisc; Trail). PAb can be used to inhibit gene expression or protein activity while purified proteins can be used to identify plant product inhibitors of enzyme activity. This is not meant to be an exhaustive list but provides important examples of how the tools of molecular biology can be used to help reduce or eliminate AFB1 contamination.

Organization and Structure of Nitrogen Utilization Genes in *Aspergillus parasiticus*: Potential Linkage to Aflatoxin Regulation

Perng-Kuang Chang[1], Kenneth C. Ehrlich[2], John E. Linz[3], Deepak Bhatnagar[2*], Thomas E. Cleveland[2] and Joan W. Bennett[1]. [1] Tulane University, New Orleans, LA; [2] USDA, ARS, SRRC, New Orleans, LA; [3] Michigan State University, East Lansing, MI.

Nitrogen regulates the biosynthesis of secondary metabolites in a variety of microorganisms. Nitrate represses the production of aflatoxin in toxigenic fungi *A. parasiticus* and *A. flavus*, whereas ammonium supports toxin production. Nitrate assimilation in fungi involves the expression of a global regulatory gene, such as *areA* in *A. nidulans*. It has also been proposed that structural genes for secondary metabolism are possibly regulated as members of a nitrogen control circuit.

A linkage was detected between the nitrate reductase gene (*niaD*) and the nitrite reductase gene (*niiA*) of *Aspergillus parasiticus*. These genes were divergently transcribed from a 1.6 kb intergenic region (*niaD-niiA*). The deduced amino acid sequence of the *A. parasiticus* nitrate reductase demonstrated a high degree of homology to that of *Neurospora crassa, Fusarium oxysporum* and other *Aspergillus* species, particularly the cofactor-binding domains for molybdenum, heme, and FAD. A portion of the deduced nitrite reductase sequence was homologous to those of *A. nidulans* and *N. crassa*. The nucleotide sequences in *niaD-niiA* of *A. parasiticus* and of *A. oryzae* were 95% identical, indicating that these two species are closely related. Several GATA motifs, the recognition sites for the *N. crassa* positive-acting global regulatory protein NIT2 in nitrogen metabolism, were found in *A. parasiticus niaD-niiA*. Two copies of the palindromic sequence TCCGCGGA, in the target site for the pathway-specific regulatory protein NIT4 in nitrate assimilation, were also identified. A recombinant protein, ANZF, containing the *A. nidulans* AREA (the NIT2 equivalent) zinc finger and an adjacent basic region was able to bind segments of *niaD-niiA* encompassing the GATA motifs. These results suggest that the catalytic and regulatory mechanisms of nitrate assimilation are well conserved in *Aspergillus*. The nitrate assimilating genes are being cloned from the *A. parasiticus* cDNA library to determine the reason for the inverse correlation between nitrate metabolism and aflatoxin production and the direct correlation of aflatoxin production with ammonium assimilation.

avnA, a Putative P-450 Monooxygenase, Is Required for the Conversion of Averantin to Averufin in *Aspergillus parasiticus*

Jiujiang Yu[1], Perng-Kuang Chang[2], Deepak Bhatnagar[2] and Thomas E. Cleveland[1]. [1] USDA, ARS, SRRC, New Orleans, LA; and [2] Tulane University, New Orleans, LA.

Aflatoxins are toxic and carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are synthesized by condensation of acetate units; their synthesis is estimated to involve at least 16 different enzymes. Recent studies show that at least 9 genes involved in the aflatoxin biosynthetic pathway are clustered within a 60-kb DNA fragment; several additional transcripts have been mapped to this gene cluster. However, the functions of the genes corresponding to these transcripts have not been elucidated. Two cDNA sequences located in between *ver-1* and *omtA* genes on the aflatoxin pathway gene cluster have been determined and tentatively named *avnA* (previously named *ord-1*) and *ord-2*. The *avnA* gene product exhibits sequence homology to cytochrome P-450 type enzymes. Disruption of the *avnA* gene in the wild-type aflatoxigenic *A. parasiticus* strain (SU1-N3) resulted in a mutant which was non-aflatoxigenic but accumulated a bright yellow pigment. Co-migration studies on TLC plates in 5 different solvent systems showed identical migration patterns of the accumulated metabolite and averantin, a known aflatoxin precursor. Southern blot analysis of the mutant indicated that the disruption occurred in the *avnA* gene. Precursor feeding studies confirmed that the block in the aflatoxin biosynthetic pathway created by the mutation resulted in the disruption of the averantin to averufin conversion step in toxin synthesis. We have, therefore, concluded that the *avnA* gene is involved in the conversion of averantin to averufin in the aflatoxin biosynthetic pathway in *A. parasiticus*.

MICROBIAL ECOLOGY

Summary: Panel Discussion on Development of Management Strategies Based on Knowledge of Microbial Ecology

The panel consisted of : R. Cole, D. McGee, M. Nelson, R. Norton, and B. Horn. The discussion was chaired by P. Cotty who also prepared this summary.
Comments were also made by J. Dorner, N. Keller, R. Lynch, P. Dowd, C. Martinson, T. Michailides, O. Modesto, D. Park, B. Sacher, T. Schatzki, D. Wicklow, N. Widstrom, D. Wilson, R. Winesca.

The discussion was initiated by Don Wicklow who asked if *Aspergillus tamarii* might be useful as a niche competitor for *A. flavus* in a manner similar to atoxigenic strains *of A. flavus*. Bruce Horn and Peter Cotty felt *A. tamarii* would not adequately compete with *A. flavus* in the field. Cotty indicated that some laboratory and greenhouse studies with *A. tamarii* had been done. Themis Michailides suggested that applying atoxigenic *A. flavus* strains along with substrates, such as certain fertilizers, may give the strains an extra boost during initial colonization of the crop. Cotty indicated a primary strategy in formulating atoxigenic strains was to apply them with nutrient sources that would fuel their initial growth and dispersal.

Dick Cole and Cotty were asked if drought conditions favor atoxigenic *A. flavus* strains over aflatoxin producers. They felt one or the other was not favored by drought and that both aflatoxin producers and non-producers had similar adaptations to heat. However, they also indicated a lack of data on direct comparisons between the two. Cole commented that determining exactly when and how the fungus invades peanuts during drought is an important topic that needs additional attention.

Cole and Cotty were questioned on how long the atoxigenic strains may last in the soil and Cotty was asked what caused the ratio of atoxigenics to aflatoxin producers to change over time in his tests. Were the atoxigenics more laboratory strains and not adapted to the environment? Cotty indicated that the strains were not "laboratory strains." Both his data on the distribution of atoxigenic vegetative compatibility groups in cotton producing areas and Bruce Horn's data (presented during the session) on the natural occurrence of an atoxigenic vegetative compatibility group indicate the strains are naturally occurring and competitive in the environment. However, Cotty indicated that some of his data may indicate atoxigenic strains do not survive, over relatively long periods of time, as well as aflatoxin producing strains in the soil when they are not colonizing a crop or other resource. He sees influences of atoxigenic strain applications 3 or 4 years after applications are made; over that period the atoxigenic may have colonized crops several times to replenish its propagule count. Cole indicated that in early studies strains stayed in the soils for three years; he felt *A. parasiticus* may have a little better ability to survive in the soil than *A. flavus*.

Cole indicated that both he and Cotty felt that the strains may be more effective when applied over a large area for several years. In such a scenario, he joked, we may be aiming at putting the aflatoxin producers into extinction in agricultural fields.

Doug Park asked if atoxigenic strains produced other mycotoxins, not previously described, that might serve in the ecological role of aflatoxins. Cole indicated that they were looking at all the known toxins in the aflatoxin pathway as well as toxins out of the pathway. He felt that because aflatoxin-producing fungi have been studied for toxin production so carefully, the probability of a new potent toxin, previously not described, in one of the atoxigenics was low.

Dave Wilson mentioned tests that he and others in the peanut breeding program had performed. They had put *A. parasiticus* out for several years (on colonized corn) in Georgia and Arizona and found *A. parasiticus* did not survive in Arizona. He commented that those results go along with Cotty's observation that *A. parasiticus* was hard to find in Arizona. He said that this may indicate the atoxigenic strains being applied should be adapted to the local environment in which they are intended to be active. Cotty agreed and said that he had four or five unpublished lines of evidence that *A. flavus* communities in different locations are distinct and locally adapted. He felt selecting strains for particular locations and/or environments may be a key to getting successful aflatoxin control and in particular long-term displacement of aflatoxin producers. Cotty indicated that he has sampled across the cotton belt and there are some differences among the fungi and in fungal adaptations.

Park further questioned if strains that do and do not produce aflatoxins compete in the same niche, should not the atoxigenics produce some toxin to replace their lack of aflatoxin production. Cotty indicated that there is great variability among aflatoxin producing fungi; this variability occurs in both physiological and morphological traits. A portion of this variability is in the toxin profile. This variability probably reflects diverse adaptations to specific ecological niches. He indicated that a strain may be adapted to multiple niches and the aflatoxin producers and the atoxigenics may not have an identical array of ecological niches. Some of the strains which produce the most aflatoxin lack the major polygalacturonase needed to degrade plant tissues and thus, these strains are poor at colonizing and degrading plant tissues. These strains are apparently better adapted to some ecological niche other than one requiring infection of a crop. Certain atoxigenics are better adapted to crops than these toxin producers. Compared to aflatoxin producers, the atoxigenics used as biocontrol agents are either as well adapted or better adapted to colonizing the crop. By introducing atoxigenic strains early, before the great increase in *A. flavus* associated with crop production, the atoxigenics are exposed to the crop and allowed to compete with toxin producers for crop resources.

Charlie Martinson asked if a new atoxigenic strain is used, would all the same testing be required of it by the EPA. Cotty indicated that the extent of testing required to use new strains would have to be negotiated with the EPA. However, some data would probably be transferable. For example, it may be possible that equivalent efficacy data would not be required for future strains.

Neil Widstrom asked Denis McGee to explain how isolations were done from different parts of corn silks. McGee deferred the question to Olanya Modesto. Modesto detailed the procedure for isolating from portions of silks either emerged from the husk, attached to the kernels or in between. Widstrom further questioned McGee about his failure to find more aflatoxin in 1995 even though the warm weather during silking had led him to suspect it would occur. He wondered if other factors besides temperature explained the lack of toxin. McGee indicated that the conditions during silking were good for infection but there wasn't adequate heat and drought prior to the silking period. McGee felt this earlier period was needed to permit an adequate buildup of *A. flavus* inoculum. Apparently, after silking, even though conditions for infection were good, there probably was inadequate inoculum to induce widespread aflatoxin contamination. Pat Dowd commented that he and Wicklow had similar experience in Illinois. In Illinois, although it was a very hot year, planting occurred on time in the area they were working, and it was very hot during milk stage, as well as, for two weeks prior and after. Corn infection by *A. flavus* was not widespread. He felt early rainfall may have prevented a bad aflatoxin year. Ten inches of rain occurred in parts of Illinois in May of 1995, but very little rain fell in May and June of 1988. McGee indicated that the fungus likely needed the high temperatures to out-compete other fungi and increase in magnitude. Wicklow indicated that the experience gained in 1995 showed clearly what happens when heat and temperature stress during kernel filling is separated from drought before kernel filling. Wicklow indicated that he wounded many ears of corn with a cork borer and at harvest these ears were not infected with either *A. flavus* or *A. niger*. However, certain fungi associated with cooler weather were present. Wicklow further pointed out that we do not know how high *A. flavus* populations got prior to the 1988 outbreak and that we do not know if the peak *A. flavus* population at harvest in 1987 may have predisposed the 1988 crop to contamination. Much of the panel and audience agreed that at harvest a huge amount of *A. flavus* can be added to the soil and that *A. flavus* from the prior season crop might predispose the following crop to contamination. McGee mentioned that the high *A. flavus* population from 1988 gradually declined over 12 months to what appears to be a maintenance level. Dave Wilson mentioned some unpublished experiments comparing dry-land and irrigated corn and peanut culture in Georgia; dry-land fields invariably had more *A. flavus* carryover to the next year than irrigated fields. Widstrom pointed out that in addition to high inoculum and other factors, a sustained high temperature, which means a high temperature at night, was needed to get the most vulnerability to contamination. If night temperature dropped, conditions were not as conducive to contamination. McGee agreed but reiterated that these high temperatures were also needed in June and July when the fungal soil populations begin to increase.

Ralph Walesca pointed out that earlier work suggested sorghum becomes infected at flowering and that the seed become uniformly infected. Cotty disagreed that *A. flavus* routinely systemically infects Sorghum. Cotty felt many Sorghum infections not occurring through insect wounds were a result of post maturity infections resulting from exposure of the crop to both *A. flavus* inoculum and adequate heat and moisture to allow *A. flavus* to infect the mature seed.

Tom Schatzki asked what the relation was between CFU (quantity of fungus on the crop) and ppb (quantity of aflatoxin in the crop). He also asked what conditions were the most important in dictating how much toxin an isolate makes. Dick Cole mentioned that very high levels of infection of peanut can occur but, if the peanuts have not come under stress, fungal growth is arrested and little aflatoxin is formed. He emphasized that the fungus has to invade and grow to produce aflatoxin in the crop. Doug Park pointed out that several researchers have found water activity to markedly influence aflatoxin production.

The similarity between Merritt Nelson's observation that a previous sorghum planting reduced *A. flavus* populations and Wilson's observation on influences of previous season irrigation was discussed. In Nelson's data, the influence was only a briefly lower population level. This led to a discussion of differences among *A. flavus* populations in different parts of the country. The magnitude of *A. flavus* populations is very different in Illinois or Iowa where Dowd, Wicklow and McGee ran experiments than in western Arizona or southern Georgia. This may mean the build up to an *A. flavus* population explosion may take much longer than in regions where population levels never drop below 40 or 50 propagules per gram of soil. Cole joked that the soil in McGee's fields was near sterile and had less *A. flavus* than the dust under his desk. Several in attendance concurred. McGee believes that this is a reason why the mid-west has few aflatoxin epidemics. But when epidemics do occur, the increase in soil populations of *A. flavus* is explosive.

Wicklow asked Cotty if *A. flavus* populations in uncultivated land in the deserts of Arizona fluctuate with season as in the cultivated land. Cotty didn't know how the native populations changed over seasons. He commented that the populations were quite low compared to cultivated land and that Nelson's data showed the populations dropped off rapidly in transitions from cultivation to desert. Cotty agreed with Wicklow that understanding the seasonal behavior of *A. flavus* in natural niches may be important.

Widstrom asked Robert Norton at what levels the compounds he evaluated occurred in corn. These were chemicals Norton had tested for toxicity to *A. flavus* and for ability to inhibit aflatoxin biosynthesis. Norton indicated he had done estimates on a few of the compounds and the concentrations were probably 1 to 20 mg/g. However, the compounds only occur in tissue layers 1 or 2 cells thick. The compounds are pigmented and occur at greatest concentration in highly pigmented corn (i.e. purple corn).

Robert Lynch asked if there was any coordination among those working on biocontrol. Lynch was concerned that if products were developed for each crop specifically that none would have a great enough market to support a product. Cotty responded that the agents don't seem to be crop specific. However, he again emphasized his feeling that strains were locally adapted and if biocontrol hoped for long-term population changes, strains should be selected for adaptations favoring survival in the target location. Cotty gave as example *A. flavus* strains that are resistant to borate being common in soils with high borate concentrations. Cole indicated that as time progresses he expected a lot more refinement in what strains are used.

Wilson asked Cole to comment on his experience using a red-brown mutant strain (one that accumulates norsolorinic acid) and how persistent the strain was. Cole indicated that it was very persistent and competitive; his group got a lot of red peanuts out of the plots. He joked it would be a good biocontrol agent except for accumulating both norsolorinic acid and aflatoxin. Cole said the strain could be observed growing in the tissue because of its colored mycelium. Joe Dorner indicated that the strain persisted in treated soil for at least 3 years. Nancy Keller, who previously had used the mutant on corn, commented on the work of a student that tried to use the same strain to evaluate peanut cultivar resistance. Cole indicated that his group had also evaluated use of the strain to study peanut infection.

Martinson asked Bruce Horn if he ever observed aflatoxin producers and atoxigenics in the same vegetative compatibility group. Horn indicated that they did not and that they saw very little variability within a group in toxin production. Cotty indicated that he has analyzed vegetative compatibility groups that have both aflatoxin producing and atoxigenic members.

Bob Sacher mentioned that atoxigenic strains of *Fusarium moniliforme* had been generated at the Peoria lab and asked if this meant atoxigenic strains of many fungi might be similarly useful. Comments were made that similar concepts may be broadly applicable but participants weren't sure if the *F. moniliforme* atoxigenics could compete with toxin producers and prevent contamination of corn with fumonisin.

Progress Towards Application of Atoxigenic Strains of *Aspergillus flavus* to Commercial Cotton

Peter J. Cotty
Southern Regional Research Center, A.R.S., U.S.D.A., New Orleans, LA

Recent reports on efficacy of atoxigenic strains in preventing aflatoxin contamination of cottonseed have stimulated industry interest in large scale applications of atoxigenic strains to the commercial crop in Arizona. Losses resulting from unacceptable aflatoxin levels differ over the cotton belt. In Arizona, losses due to aflatoxin contamination have been particularly severe in the 1994 and 1995 crop years. With the help of Dr. Christina Hartman of the IR-4 Biopesticide Program, we have applied to the U. S. Environmental Protection Agency for an Experimental Use Permit. If granted, the permit will allow treatment of limited commercial acreage in Arizona during the 1996 cotton season.

Economics of aflatoxin contamination will probably dictate the regions in which atoxigenic strains are utilized. We hope to produce materials for atoxigenic strain applications for $5.00 per acre or less. If treatments are 70% effective and an average of 40% to 70% of seed is above 20 PPB and the benefit of having aflatoxin free seed is $20 to $40 a ton then growers will gain an average return above an initial $5/acre investment of $0.60/acre to $14.60/acre. Economics may be improved by long-term benefits resulting from strain ability to remain in fields until the next crops are planted. Benefits may also arise from the applied atoxigenic strains remaining with the crop until use and thus preventing increased contamination during transit and in storage.

Just as dust doesn't stay in the field in which it is raised, fungi do not stay in the field to which they are applied. Thus, over time, applications may reduce contamination in an area as a whole. This may facilitate the development of community wide management programs. In areas where multiple crops are affected by contamination (*i.e.* corn, cotton, and peanuts), treatments to one crop may benefit all crops. The economics of applications in such areas may be complex.

Development of a product based on atoxigenic strains and sold as an agrochemical would probably be the simplest course to producing an aflatoxin control product. However, the initial market for atoxigenic strain products may be too small to warrant significant investment by an agrochemical company. Alternatives to development by an agrochemical company may include development of Pest Control Districts. Advantages of such programs may include tailoring to specific regions, increased cost effectiveness, and development of mechanisms for funding the monitoring of fungal populations.

Regardless of the means of intervention employed, there will be fungi associated with crops. Dead, weakened, and partially decayed plant tissues are readily available in agricultural environments, and it is not feasible to prevent utilization of these resources by fungi. A level of control over which fungi become associated with crops may be permitted by seeding select fungal strains into agricultural fields.

The Influence of crop rotations on soil surface populations of *Aspergillus flavus* in Arizona in the context of regional and within-field spatial patterns

Merritt R. Nelson[1], Donna M. Bigelow[1], Thomas V. Orum[1], Donald R. Howell[2], and Peter J. Cotty[3]

[1] Department of Plant Pathology and [2] Cooperative Extension Service, University of Arizona, [3] Southern Regional Research Center, USDA, ARS.

Understanding the strain composition of soil surface populations of *Aspergillus flavus* is important to aflatoxin management because some strains are capable of producing more aflatoxin than others. *A. flavus* isolates can be broadly grouped as S strains and L strains based on the presence and size of sclerotia. As a group, S strain isolates are much more toxigenic than L strain isolates and, therefore, areas in which the percentage of S strain isolates is high are at greater risk of aflatoxin contamination than areas in which the percentage of S strain isolates is low.

In August 1993, sixteen commercial fields in Yuma County, Arizona, were selected for a study of the effects of crop rotation on total propagule counts and percentage of S strain isolates. In March 1995, fourteen fields were added to the study so that regional spatial patterns could be explored along with crop rotation. The thirty fields were arranged in nine groups from Texas Hill to San Luis. A nested sampling design was used to increase the chance of showing patchiness at several scales from 10 m to more than 10 km. Samples were collected in March, July, and October 1995 and assayed for total *A. flavus* propagule counts and percentage of S strain isolates by dilution plating. Results from the March and July sampling indicate significant differences among groups of fields in the percentage of S strains. There was also a recurring spatial pattern in which one group was consistently high in percent S in August 1994, March 1995, and July 1995. The percentage of isolates classified as S strains was significantly higher in July 1995 (60% of 3773 isolates) than in March 1995 (40% of 2077 isolates). The July 1995 result is consistent with the August 1994 result (59% of 803 isolates from 16 fields were S strain). No regional patterns (differences among groups) were seen for total propagule counts, but there were significant differences among fields within groups. This is consistent with field conditions playing an important role in total propagule counts. In July 1995, twenty five of the thirty fields were in cotton and averaged 1010 propagules per gram compared with a field of mature wheat that averaged 71 propagules per gram. Within field variability is high for both propagules/gram and percentage of S strain isolates, so a relatively large number of samples is required to reveal patterns. In this context of high within-field variability, the dominant overlaying patterns emerging so far are regional effects for percent S and field specific effects for total propagules per gram.

Results from our crop rotation experiment at the Maricopa Agricultural Center (MAC) in Pinal County, Arizona are consistent with the conclusions from Yuma County that field conditions strongly influence propagules per gram but not percentage of S strain isolates. The MAC experiment consists of sixteen 4 acre experimental plots with four treatments arranged in four blocks. Treatment 1 is solum barley was followed by cotton with minimum tillage. Treatment 2 is clean winter fallow (no barley) followed by cotton using standard practices. Treatment 3 is solum barley plowed under as green manure for cotton. Treatment 4 is solum barley followed by cotton using standard preparations (in 1993 and 1995) or by sorghum (in 1994). In October 1994, total propagule counts for treatment 4 (sorghum) were 409 propagules per gram compared with 7966, 2831, and 4440 propagules per gram for treatments 1, 2, and 3 respectively - all of which were in cotton. In May 1995, treatment 1 (minimum tillage) had significantly higher counts than the other three treatments with 81 propagules per gram, compared with 23, 43, and 42 propagules per gram for treatments 2, 3, and 4 respectively. No differences among the four treatments were observed in the percentage of S strain isolates found. The percentage of S strain isolates averaged over all four treatments was 22% in both October 1994 and May 1995.

8 th ANNUAL AFLATOXIN ELIMINATION WORKSHOP

ATLANTA, GA. OCTOBER 23-24, 1995

Epidemiological basis for control of Aspergillus flavus resistance and management.

Denis C. McGee, M. Olanya, and L. H. Tiffany, Departments of Plant Pathology, Botany, and the Seed Science Center, Iowa State University, Ames, IA 50011.

Three resistant and two susceptible hybrids, as determined by Dr. D. White, University of Illinois, were exposed to natural inoculum of *Aspergillus flavus* from waste corn obtained from a bin site in Iowa and applied to field plots just before silking at 0, 10, and 50 kg/plot. Results showed that a good relationship existed between waste corn amounts and spore discharges. Husk infection was similar across hybrids, but both silk and kernel infection were lower for the resistant compared to the susceptible hybrids. Results of kernel infection in this experiment also were in complete agreement with those obtained in an artificial inoculation experiment at the same location, thus indicating that the waste corn method is an effective natural resistance screen

In a growth chamber study, single plants of resistant and susceptible hybrids were grown in pots in a greenhouse, then transferred to growth chambers maintained at 30 C. One set of pots of each hybrid had 2 kg of waste corn, naturally infested with *A. flavus*, placed on the soil surface and another set was left untreated. *A. flavus* infection of was similar for resistant and susceptible hybrids, but silk and kernel infection were all less for resistant compared to susceptible hybrids. This experiment confirmed data evaluating resistance in the field experiment. It also demonstrated the impact of airborne inoculum on plant infection.

The second area of emphasis in the 1995 study was to examine more closely the role of soil as an inoculum source for *A. flavus*. Surveys of fields in Iowa confirmed the consistency of the July surge in soilborne population of *A. flavus* that had been found since 1993. In a laboratory study to determine the mechanism of increases in *A. flavus* population in soil, field soil was subjected to a range of temperature conditions on a thermogradient plate and periodically measured for populations of *A. flavus*. Results show that soil temperatures in the range 30-39 C greatly increased soil populations of *A. flavus* within 1 week of incubation and that this increase continued over time. It also was shown that condia of *A. flavus* were released into the air and that spore release was related to the soil-borne population. Further data showed that *A. flavus* survives and grows on small particles of corn and soybean crop residues in the soil.

Considerable progress was made in 1995 in adding information to the model that expressed linkages between soilborne inoculum and aflatoxin contamination of resistant and susceptible hybrids. These included the finding that: *A. flavus* is released into air directly from soil; silk and kernel infection is related to airborne inoculum; and resistance is associated with the silk infection pathway.

With further elucidation of this model, it may be possible to manage the disease by a combination of disease resistance and control of the soilborne population of *A. flavus* either during the build-up of the soilborne population in the spring and early summer or by reducing the soilborne inoculum below economic thresholds by cultural, chemical, or biological means.

UPDATE ON BIOLOGICAL CONTROL OF PREHARVEST AFLATOXIN CONTAMINATION OF PEANUTS

R. J. Cole, J. W. Dorner, and P. D. Blankenship, National Peanut Research Laboratory, USDA-ARES, Dawson, Ga. 31742

The use of non-aflatoxin producing strains of *Aspergillus parasiticus* and *A. flavus* as biocompetetive agents to help control preharvest aflatoxin contamination of peanuts has showed promise as part of an integrated aflatoxin management strategy. Environmental control plot studies for crop years 1994-95 were designed to determine the effect of application rate of biocompetetive agents on aflatoxin contamination of peanuts. Rice infected with non-toxigenic color mutants of *A. parasiticus* and *A. flavus* was applied approximately three weeks after planting at rates of 0, 20, 100 and 500 lbs/acre with three replicated plots per treatment. Soil analyses for determining the levels of mutants and wild-type fungal populations were conducted preplant, mid-growing season and at harvest. These data are not yet complete. Aflatoxin analyses were conducted on commercial peanut size categories. Aflatoxin results showed a treatment-related effect for both 1994 and 1995 crop years. 1994 CY peanuts from the 20, 100, and 500 lbs/acre treatments averaged 74, 35, and 33 ppb of aflatoxin, respectively. Peanuts from untreated control plots averaged 337 ppb aflatoxin. In 1995 the aflatoxin values were 184, 36, and 0.4 ppb, respectively with controls averaging 718 ppb. The data showed good aflatoxin control at all inoculum levels, but the highest level, as might be expected, showed excellent control.

Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii*

B. W. Horn, R. L. Greene, V. S. Sobolev, J. W. Dorner, J. H. Powell, and R. C. Layton

National Peanut Research Laboratory
USDA, ARS
Dawson, GA 31742

Vegetative compatibility groups within populations of *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii* from soil and peanut seeds in a peanut field were examined for differences in morphology (colony color and sclerotium characters) and mycotoxin production (aflatoxins, cyclopiazonic acid, and kojic acid). *A. tamarii* was divided into types A and B based on morphological differences and the lack of vegetative compatibility between the two types. Using digital color image processing, the four taxa were easily distinguished by colony color through analyses of peak color intensities for red, green, and blue. Color comparisons of *A. flavus* vegetative compatibility groups were not possible because of poor sporulation by many of the isolates. *A. parasiticus* group 1 differed significantly from groups 2-9 in colony color, and groups 1-3 of *A. tamarii* type A and groups 1-3 of *A. tamarii* type B were also significantly different within each type. Color image processing of filtered conidia indicated that the color difference of *A. parasiticus* group 1 was due primarily to the flocculose texture of the colony whereas group differences in *A. tamarii* types A and B were the result of conidium pigmentation. *A. flavus* and *A. parasiticus* showed significant differences among groups in number of sclerotia, sclerotium volume, and sclerotium shape (length/width ratio). Isolates of *A. tamarii* type B often produced irregularly shaped sclerotia; type A isolates were nonsclerotial. Among the 11 groups of *A. flavus*, significant differences were detected in total aflatoxin (aflatoxins $B_1 + B_2$), cyclopiazonic acid, and kojic acid. *A. parasiticus* groups also showed significant differences in total aflatoxin (aflatoxins $B_1 + B_2 + G_1 + G_2$), ratio of $G_1 + G_2/B_1 + B_2$, and kojic acid; cyclopiazonic acid was not produced by *A. parasiticus*. Nonaflatoxigenic isolates of *A. flavus* and *A. parasiticus* were restricted to certain groups and in *A. parasiticus*, all nonaflatoxigenic isolates accumulated *O*-methylsterigmatocystin, an immediate precursor of aflatoxin B_1. Isolates of *A. tamarii* type A produced cyclopiazonic acid and kojic acid whereas those of type B produced only kojic acid at concentrations six-fold higher than type A; few differences in mycotoxin production were detected among groups in the two types of *A. tamarii*. The high proportion of variation among isolates accounted for by vegetative compatibility groups suggests that isolates within groups are closely related.

SOIL FUNGI OF SOME LOW DESERT COTTON FIELDS AND THEIR ABILITY TO INHIBIT *ASPERGILLUS FLAVUS*. M.A. Klich, and C.C. Chu, U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans LA 70124 and USDA ARS Western Cotton Research Laboratory, Brawley CA.

Aspergillus flavus chronically infects cotton bolls and seeds in the desert southwest and forms aflatoxin, the most potent naturally-formed carcinogen, in the seeds. It is presumed that the soil is a major source of inoculum. In this study, dilution plates of soils from cotton fields in southwestern Arizona and southeastern California assayed for filamentous fungi, resulted in isolation of approximately 40 taxa. Methylene chloride/acetone (1:1) extracts from strains of each taxon were screened for activity against *A. flavus* by overlaying developed thin layer chromatographic plates with agar and an *A. flavus* spore suspension and by paper disk bioassay on seeded petri plates. Several strains of *Fusarium solani*, *Penicillium vinaceum* and *A. auricomus* were found to inhibit *A. flavus* germination and growth.

Elimination of Aflatoxin in Tree Nuts: Control of *Aspergillus flavus* by Saprophytic Yeasts

Sylvia S. Hua, J. Baker and O.K. Grosjean, USDA, ARS, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710

In Caliifornia, 13% of the pistachio, 10% of the almond, and 6% of the walnut harvests are contaminated with aflatoxins. *A. flavus* particularly infects tree nuts which have been damaged by insects, animals, early splits and mechanical harvest. *A. flavus* on the surface of the tree nut fruits may develop a quiescent or latent infection possibly through the cracking and the vascular tissue into the kernel. Aflatoxin-contaminated nuts are often indistinguishable from aflatoxin-free nuts and any detectable amount of aflatoxin in the nut is an indication of an established infections of *A. flavus*. Warm temperature and high humidity provide conditions for rapid accumulation of aflatoxin in the harvested nuts.

In recent years, there is a shift from the use of antibiotic-producing bacteria toward the development of non-antibiotic-producing antagonistic yeasts for the biological control of several fungi. Yeasts which can colonize a surface for a very long period under dry conditions, produce extracellular polysaccharides that enhance their survivability and restrict both colonization sites and nutrient flow to other fungi on the phyllosphere.

We have isolated a number of yeasts from the surface of pistachio, almond, apple and lemon for the screening of an effective antagonist. These yeast strains were obtained from the fruit surface by washing and sonicating. Each isolate was tested by scoring for its ability to inhibit germination, growth and sporulation of *A. flavus*. Several strains of yeast were found to be effective antagonists to *A. flavus* on PDA agar plates. Aflatoxin production as well as growth of *A. flavus* were reduced. The yeast, *Pichia guilliermondii* US 7 fastidiously attached to fungal hyphae and inhibited sporulation dramatically. Selected yeast strains were tested on wounded pistachio fruits. The spreading and sporulation of *A. flavus* were inhibited in the presence of yeasts. The total number of spores of *A. flavus* on the nuts was reduced by 30 fold when selected yeast strains were used as antagonists.

These yeast antagonists have a complex mode-of-action involving both nutrient competition and direct parasitism. The development of resistance to such antagonists should be more difficult than to those having a simpler mode of action such as antibiotic production. Thus, a cumulative biocontrol of *A. flavus* by yeast should be achieved by decreasing the population of *A. flavus* and suppressing its infection of nut kernels over several growing seasons to eventually reduce aflatoxin in tree nuts.

CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND PLANT FUNGAL RELATIONSHIPS

themis\aflatox\paneldis.95

Summary: Panel Discussion on 'Altering Agronomic Practices to Improve Management of Aflatoxin Contamination'

The panel consisted of: R. Lynch, W. Batson, T. Schatzki, T. Isakeit, and G. Simmons. The discussion was chaired by T. J. Michailides, who also wrote this summary from the discussion of the panel. Drs. J. Dunlap and D. Park did not participate in this panel discussion although their names were included in the initial schedule. Comments were also made by P. Cotty, G. Obenauf, M. Doster, D. Wicklow, and J. Dunlap.

At the beginning of the panel discussion the chairman of the session summarized some of the highlights of research presented by the participants in this panel.

First, Michailides (Dept. of Plant Pathology, University of California, Davis / Kearney Agricultural Center) reported all the accomplishments in the last 5 years' research on pistachio, fig, and walnut. These accomplishments included in pistachio: 1) The determination of source (male inflorescences) of sporulation of aflatoxigenic and other *Aspergillus* fungi in pistachio orchards. 2) The discovery of a critical irrigation time (mid-May) that substantially reduces the incidence of early splits, the main source, of preharvest aflatoxin contamination. 3) The discovery of the suture stain of early splits previously unknown to the industry that can be used to remove early splits during processing. 4) The discovery that yellow stain is not associated with molds or *Aspergillus* contamination (including the aflatoxigenic fungi). 5) Regardless of the extent of the surface brown stain on pistachio shells, increasing levels of suture staining resulted in increasing levels of mold contamination (including *Aspergillus* spp.) in the shell, the kernel, and lower kernel quality due to increasing discoloration. Based on these accomplishments recommendations have been developed for the growers and the processors to reduce preharvest aflatoxin contamination in pistachio.

The accomplishments in figs were: 1) A source of contamination was determined (figs rehydrated by irrigation). 2) The effect of sweeping that acts as a natural inoculation procedure for the figs that are still on the trees was evaluated. 3) The effect of drip irrigation (amounts of water applied as summer irrigations) on contamination of figs by *Aspergillus flavus* and other *Aspergillus* fungi. 4) Incidence of *A. flavus* and BGYF decreased with increasing amounts of applied irrigation water. 5) Characteristics of figs were determined that can help to identify and

separate contaminated figs (water soaking around the ostiole). Relevant recommendations have been developed for both growers and processors of figs.

In walnuts, more *Aspergillus* and other molds were associated with a) longer stem-end opening, b) longer-sized nuts, c) more navel orangeworm infestation, d) more early husk splits, e) longer periods walnuts remained on the ground, and f) more sunburn. The conclusion of this 5-year mid-season summary is that all these accomplishments of research on pistachio, fig, and walnut not only provided information on the basic ecology of the aflatoxigenic fungi in tree nut and fig orchards, they also helped directly towards the goal of reducing aflatoxin in these crops.

Dr. Lynch (USDA/ARS, IBPMRL, Tifton, GA) reported on the influence of insects on aflatoxin contamination in peanut and activity of transgenic peanut against the lesser cornstalk borer (LCB). He mentioned that, in a randomized complete block design with 5 various treatments involving insecticide applications against the lesser cornstalk borer, he found little effect on aflatoxin contamination in peanut kernels under the severe drought conditions of the test. In one of the two years of the study, Southern Runner cultivar had a significantly higher level of aflatoxin contamination than did Fluorunner. Age of peanuts at harvest and degree of lesser cornstalk borer damage to pods significantly affected aflatoxin contamination in all 3 years.

Dr. Lynch also summarized the results of his laboratory in evaluating transgenic peanut plants containing a modified gene from *Bacillus thuringiensis* for activity against LCB. Bioassays using peanut leaflets from regenerated plants of certain lines showed high toxicity and those of other lines showed moderate toxicity to 7-day larvae of LCB. He concluded that peanut lines T26-1-12, T26-11-53, T26-12-3, and T30-9-140 contained sufficient insecticidal activity due to the expression of the modified *CrIA (c)* gene to justify evaluation against other peanut insects.

Dr. Batson (Dept. of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS) reported on the incidence of *Aspergillus flavus* seed infection and aflatoxin contamination in Mississippi cotton modules and the influence of field storage. He summarized the survey results from 118 modules, detecting *A. flavus* in 20% of the samples and low amounts of aflatoxin in 21% of the *A. flavus*-positive samples. In modules constructed from inoculated bolls in an experiment, he showed that aflatoxin increased over time in one of the modules. This relationship was not true in the second module. The amounts of aflatoxin of samples taken from

the exterior of the modules were similar to those found within the modules and there was no significant change over time.

Dr. Schatzki (USDA/ARS, Albany, CA) reviewed the flow chart of processing pistachio nuts and explained the various categories of nuts and the distribution of the contaminated nuts. He also explained the frequency, size of samples, and amounts of contamination of nuts. He measured samples from 19 product streams and showed that sorting can remove 89% of aflatoxin by removing 5% of the product. According to Schatzki, sorting of pistachios has been improved by recovering 2/3 of "bad" nuts. He has developed a model which shows that nuts with 10^3 - 10^6 ppb represent early splits. He also reported on the x-ray imaging for detecting insect-infested nuts. The distribution of alfatoxin in almonds, a study supported partly by the California Almond Board Dr. Schatzki said, represents only 1/4 of the ppb found in pistachios. Sorting in almonds can emphasize concealed damage and agricultural engineers at the University of California, Davis, are working on instrumentation for spectral recognition of these nuts.

Dr. Isakeit (Texas A& M University, Weslaco, TX) reported on the relationship of insect injury and cotton root rot to aflatoxin contamination of cottonseed in South Texas, a project in cooperation with Dr. Dunlap. They studied two possible predisposing factors to aflatoxin contamination of cottonseed in South Texas: 1) cotton root rot caused by *Phymatotrichiopsis omnivora* and 2) insect injury. They showed that there was no apparent relationship between cotton root rot and aflatoxin contamination and concluded that cotton root rot is not a predisposing factor to aflatoxin contamination. In contrast, they showed that insect injury of the boll prior to maturity was an important factor for aflatoxin contamination in South Texas based on samples collected from seven gin yards. Interestingly, 79% of the module and gin cart samples had BGYF, with aflatoxin ranging from 0 to 218,750 ppb. Dr. Isakeit concluded that these highly-contaminated seed samples may cause one and a half pounds of noncontaminated cottonseed to exceed the allowable aflatoxin limit.

The last speaker of the Tuesday afternoon session, Dr. Simmons (USDA/ARS, Fresno, CA) reported on a hydrogen peroxide treatment of pistachios to reduce aflatoxins and microbes. A 10% alkaline hydrogen peroxide solution was used to reduce aflatoxin in pistachio meats. Although the aflatoxin concentration appeared to be reduced by both alkaline hydrogen peroxide or an alkaline water washing, no statistical difference was found between the treatment and the control. (The aflatoxin concentration of pistachios meats 3 and 7 days after inoculation was

approximately 3,000 and 30,000 ppb, respectively.) The use of vapor hydrogen peroxide at a concentration of 3 mg/liter volume air for 5 minutes was very effective in reducing the load of microbes on pistachio nut meats. The vapor hydrogen peroxide treatment should be considered a potential replacement if the registration of propylene oxide is revoked as presently proposed. The use of hydrogen peroxide, a Generally Recognized As Safe Substance under present Food and Drug Administration regulations, would require at least a self affirmation of GRAS.

Because there was no time for specific questions after each presentation, the chair of the panel asked that specific questions related with the material presented should be directed to the presenters first and then the discussion would be open to more general matters related with the topic of the panel.

Cotty asked Michailides whether it is economical to have extra irrigations in order to prevent contamination. Michailides responded that it may be economical, especially when increased yields can accompany well irrigated fig orchards. Doster said that the total increase in irrigation water applied represents a few inches of water over the total water requirements of the trees. Cotty also mentioned that perhaps detection of BGYF within figs using an intense directed light and appropriate filters with a photomultiplier could be possible. Michailides agreed that was possible and he might need to further look into it .

Wicklow asked Batson what he meant by mentioning twice the word frustration during his presentation. Batson explained that in order to study the influence of storage of seed cotton over time in modules on *A. flavus* seed infection and subsequent aflatoxin contamination, one needed a contaminated module. Levels of seed infection and aflatoxin contamination to date have been very low.

Cotty asked Lynch what the mechanisms were of the increase in toxin he saw when he left the peanuts in the ground. Lynch said he felt it was an increase in toxin in already infected peanuts and not new infections. Adding to his initial question, Cotty asked if the peanuts matured more while they awaited harvest, and Lynch replied that they did.

Lynch asked Michailides about the association of fig wasp (*Blastophaga psenes*) that is needed for the pollination of certain fig cultivars. Michailides explained that although the wasps are not associated with contamination or spread of *Aspergillus,* he mentioned that he occasionally

isolated *Aspergillus niger* from fig wasps. However, he said, we usually isolate *Fusarium moniliforme* that is spread by fig wasps and cause fig endosepsis in figs, a very damaging disease of figs. Michailides explained that when wasps enter the figs to pollinate them, the figs are still very small and green, a stage which is not susceptible to *Aspergillus s*pp. He also mentioned that fig wasps are the main carriers of *Fusarium moniliforme* and various yeasts but have no evidence on ways that wasps actually contribute to any of the *Aspergillus* spread. Doster also added that although occasionally *Aspergillus niger* is found in green figs, the incidence is substantially less than that recorded later when figs are mature, which suggests the wasps are not important.

Obenauf questioned Michailides about his research on the irrigation effects on *Aspergillus* section *Flavi.* He mentioned that since 90% of the orchards are irrigated by flooding, the drip irrigation studies may have no application in the majority of the orchards. Interestingly, Michailides pointed out that it is the orchards irrigated by drip that usually have the problems because of the fact that a lot of figs land under the drip lines and get infected by *Aspergillus* species, including the aflatoxigenic fungi. Michailides disagreed with Obenauf 's percentage of fig orchards irrigated by flooding because he believed that the acreage of fig orchards irrigated by flooding was much much smaller now, since all the new acreage planted is under drip irrigation. He also mentioned that subsurface irrigation is a way to avoid the aflatoxin problem because growers can provide the necessary amounts of water for the fig trees and at the same time not have the surface of the soil wet. He pointed out that although converting the old orchards to this system is not easy and seems impractical, at least this method of irrigation is something the growers have to consider when they plan for new plantings.

Michailides asked Isakeit if he noticed any natural cracking of the cotton bolls as they matured, which may explain how *Aspergillus flavus* can invade cotton bolls. Isakeit explained that this is possible especially when the bolls get dry as they mature. Cotty agreed that indeed bolls can have natural cracks as they mature and also he pointed out the possibility of microwounds created by various insects attacking cotton.

Batson was asked by Michailides whether his low levels of aflatoxin in the inoculation experiments can be explained by the use of an isolate that actually was not "hot." Batson said that the isolate was obtained from Cotty and referred to him for additional information. Cotty admitted that the isolate he had provided might not be one of the "hottest" ones.

Wicklow asked Lynch about the non-association of insects and aflatoxin contamination in peanut. Lynch explained that under the extreme environmental conditions in the plastic-covered, rain-out shelters, Lorsban activity dissipated rapidly and was insufficient to control lesser cornstalk borer damage to peanut pods. As damage to the pods increased and the pods remained in the soil, aflatoxin accumulated in kernels from all treatments. Initially, there was a significant difference in aflatoxin concentration between pod damage classes with the most damaged pods having a higher level of aflatoxin. But, as the length of the time pods were exposed to drought increased, fewer differences were noted between pod damage classes.

Aflatoxin Control in Pistachio, Walnut, and Figs: Identification and Separation of Contaminated Nuts and Figs, Ecological Relationships, and Agronomic Practices.

M. A. Doster, T. J. Michailides, and D. P. Morgan, Dept. of Plant Pathology, and D. A. Goldhamer, Dept. of Land, Air & Water Resources, Univ. of California, Davis / Kearney Agricultural Center, Parlier 93648.

Pistachios. Our objective was to investigate how shell staining was related to poor kernel quality. Nuts from a commercial processor were separated into various categories according to the amount of general shell staining and the amount of suture area stained, which is characteristic of "early split" pistachio nuts (the major source of preharvest aflatoxin contamination). The kernels were examined for fungal infections, discoloration, and insect infestation. No sign of fungal growth was observed on kernels of nuts with no shell discoloration. For nuts with limited general shell staining (1-10% of the shell surface stained), the amount of the suture stained indicated the likelihood of kernel decay. For example, nuts with 1-10% of the suture stained had 16% of the kernels decayed, whereas nuts with more than 64% of the suture stained had 31% kernel decay. Most nuts with severe general staining (>10% of the surface) and extensive suture staining (>36% of the suture) had kernel decay. Our results suggest that external shell staining can be used to remove nuts with poor quality kernels.

Walnuts. The evaluation of walnuts from 1994 harvests was completed. The following types of nuts were associated with higher than normal levels of kernel infection by *Aspergillus* spp. or other fungi: sunburned nuts, nuts infested with navel orangeworm, nuts on ground before harvest, nuts with earlier husk split, nuts with larger openings at the stem end, and larger sized nuts. The evaluation of walnuts from the 1995 harvests is in progress.

Figs. Further studies on the use of bright greenish yellow (BGY) fluorescence for removing contaminated figs were performed. The following species were isolated from 71 Calimyrna figs that showed BGY fluorescence (in parentheses is the percentage of isolates for the specified species): *A. tamarii* (41%), *A. flavus* strain L (39%), *A. flavus* strain S (17%), *A. parasiticus* (1%), and *A. alliaceus* (1%). Calimyrna figs in a research orchard were inoculated with fifteen isolates in *Aspergillus* Section *Flavi*. For all isolates, infected figs showed BGY fluorescence, even though six isolates were originally from figs that did not show BGY fluorescence. In addition, two isolates of *A. nomius* also produced the typical BGY fluorescence.

Calimyrna figs were inoculated with *A. flavus* in a research orchard on two dates in August 1995 in an experiment similar to one performed in 1994. Two weeks after inoculation, figs were examined for infection by *A. flavus*. Figs became more susceptible as they matured through the four developmental stages (in parentheses are percentages of figs with external and internal *A. flavus* colonization, respectively): green with eye closed (0%, 0%), green with eye open (4%, 1%), yellow (18%, 20%), and brown (54%, 71%). Aflatoxin analysis of the figs from the 1994 harvest were completed and showed that brown figs had more than four times the aflatoxin of yellow figs and more than 25 times that of green figs. In addition, artificial wounding of figs in the research orchard resulted in more infections by *A. flavus* for green figs with the eye open and yellow figs, although very immature figs (green with eye closed) and very mature figs (brown) had the same number of infections for nonwounded and wounded figs. In a laboratory experiment, green figs with closed eyes after artificial wounding became susceptible to infection by *A. flavus* and *A. parasiticus*.

Studies were initiated to determine if the new cultural practice of irrigating Black Mission orchards during the harvest period would result in more mold and aflatoxin contamination. Figs on wet soil were substantially more moldy than figs on dry soil, even when the figs were on wet soil for as short as two days before harvest. However, the levels of *Aspergillus* spp. were very low, and further studies are needed.

Irrigation treatments ranging from 75 to 225% of normal water requirements were applied in a Black Mission and in a Calimyrna fig orchard. In the Black Mission orchard, very few figs were infected with *Aspergillus* spp. However, in the Calimyrna orchard the incidence of fungi in *Aspergillus* Section *Flavi* and incidence of figs with BGY fluorescence decreased with increasing amounts of applied water in the summer. These results suggest that figs might be similar to such crops as peanuts and corn, where drought stress results in greater aflatoxin contamination.

Publications in 1994-95:

1. Doster, M.A., and T.J. Michailides. 1994. Development of *Aspergillus* molds in litter from pistachio trees. Plant Disease 78:393-397.

2. Doster, M.A., and T.J. Michailides. 1994. *Aspergillus* molds and aflatoxins in pistachio nuts in California. Phytopathology 84:583-590.

3. Doster, M.A., and T.J. Michailides. 1995. Characteristics of pistachios with high incidence of mold and navel orangeworm contamination for identification at processing plants. Pages 67-69 in: Calif. Pistachio Industry Annual Rep. Crop Year 1994-95, Fresno. 144 pp.

4. Doster, M.A., and T.J. Michailides. 1995. The relationship between date of hull splitting and decay of pistachio nuts by *Aspergillus* species. Plant Disease 79:766-769.

5. Doster, M.A., and T.J. Michailides. 1994. Factors involved in the development of kernel mold in walnut. Pages 342-345 in: Walnut Res. Rep., Walnut Marketing Board, Sacramento. 367 pp.

6. Doster, M.A., T.J. Michailides, and D.P. Morgan. 1995. *Aspergillus* species and mycotoxins in figs from California orchards. Plant Disease 79: (accepted).

7. Doster, M.A., T.J. Michailides, and D.P. Morgan. 1995. Aspergillus molds and aflatoxins in figs. Fig Annual Res. Rep. Crop Year 1994-95. California Fig Institute, Fresno. 9 pp.

8. Michailides, T.J., M.A. Doster, D.P. Morgan, and D.A. Goldhamer. 1995. Effect of summer irrigation on contamination by molds of Calimyrna and Black Mission figs. Fig Annual Res. Rep. Crop Year 1994-95. California Fig Institute, Fresno. 9 pp.

FACTORS INVOLVED IN THE DEVELOPMENT OF *ASPERGILLUS* SPP. AND OTHER MOLDS IN KERNELS OF WALNUTS

Mark A. Doster and Themis J. Michailides. Department of Plant Pathology, University of California, Davis / Kearney Agricultural Center, Parlier 93648.

In order to identify preharvest factors associated with fungal colonization of walnut kernels, the kernels of over 4,000 walnuts were collected from a research orchard (cv. Chico) and evaluated for kernel decay. One question we wanted to answer was whether the walnuts on the ground before harvest ("windfalls") had more mold contamination than those still on the tree ("shaken"). The walnuts that were on the ground 15 days before harvest did have substantially more kernel colonization by *A. niger* than the other walnuts, but only slightly more by other fungi and only slightly more insect infestation. From these results it is not clear that windfalls have sufficiently higher levels of kernel mold to recommend any specific treatment by growers. However, walnuts that had husks split 15 days before harvest had almost twice the amount of kernel contamination by *A. niger*, other fungi, and insects compared to walnuts that split their husks closer to harvest. These results suggest that the earlier the husk splits, the more likely that the kernel is colonized by molds.

Several preharvest factors or characteristics were found to be associated with fungal colonization of the walnut kernel. Insect infestation (predominantly navel orangeworm) resulted in high levels of fungal contamination of the kernel, although infestation was not necessary for kernel decay to occur. Walnuts that had the husk shriveled and attached to the shell (at least partially) had more than triple the fungal contamination and insect infestation of the other walnuts. The shriveled attached husk would be a characteristic that could be used during processing to remove contaminated nuts. Another useful characteristic could be the size of the hole at the stem end of the nut, because the larger the size of the stem-end hole, the more fungal contamination and insect infestation of the kernel. An interesting discovery was that the larger the size of the walnut, the higher the incidence of fungal colonization of the kernel. Finally, sunburned walnuts had more kernel contamination by *A. niger*, by other fungi, and by insects compared to normal nuts. Our results suggest that the husk plays an important role in protecting the kernel, and when the husk is damaged (i.e., sunburned) or splits, then the kernel is at risk for fungal decay.

Postharvest factors involved in fungal colonization of walnuts are poorly understood. We observed colonization by six *Aspergillus* spp. including *A. flavus* of packaged in-shell walnuts. Two possible factors that could explain this fungal colonization are packaging inadequately dried walnuts (moisture content too high) or storing the packages under conditions that could result in condensation and rehydration of the walnuts.

CONCLUSIONS

Several characteristics (shriveled husk, size of stem-end hole, nut size) were associated with kernel mold. Some of these characteristics might be useful for removal of contaminated nuts during processing. In addition, several preharvest factors (early husk split, insect infestation, sunburn) were associated with kernel decay. By controlling these factors the amount of kernel mold might be reduced (for example, reducing navel orangeworm infestation should reduce fungal colonization of the kernel). Postharvest colonization of walnuts by *Aspergillus* fungi can occur, but the factors involved are poorly understood. Although the results presented here represent preliminary results for a multiple year study, it is clear that the kernel mold situation for walnuts is very complicated.

Influence of Insects on Aflatoxin Contamination in Peanut and Activity of Transgenic Peanut Against the Lesser Cornstalk Borer

R. E. Lynch, D. M. Wilson, and P. Ozias-Akins
USDA-ARS, IBPMRL, Tifton, GA; University of Georgia, Department of Plant Pathology, and University of Georgia, Department of Horticulture, Tifton, GA, respectively

Insects and insect damage have often been associated with aflatoxin contamination in peanut for years. However, until recently, only pods that had been penetrated by the lesser cornstalk borer (LCB) were considered at risk for aflatoxin contamination. Research was conducted at Tifton, Georgia, to determine the extent of insect damage necessary to enhance aflatoxin contamination in peanut. The research was conducted in 1992-1995 using moveable greenhouses to induce drought and an epidemic of LCB. In 1992, Florunner was evaluated, and in 1993-1994, Florunner and Southern Runner were evaluated. Peanuts were planted in plots 24.4 meters long and 5 beds wide with 2 rows/bed in each of 4 replications. Treatments for control of the LCB were 1) an untreated control; 2) chlorpyrifos applied at planting; 3) chlorpyrifos applied at pegging; 4) chlorpyrifos applied at planting and at pegging; and 5) an IPM application of chlorpyrifos (i.e. just before initiation of an LCB epidemic). The experiments were designed in a randomized complete block with 4 replications and a split-split plot arrangement of treatments in 1992 and a split-split-split plot in 1993-1994. In 1992, whole plots were insecticide treatments and subplots were age at harvest. In 1993-94, insecticide treatments were whole plots, peanut varieties were subplots, and age of the peanuts at harvest were sub-subplots. Optimum peanut production practices were employed from planting through 90 days, after which the greenhouses were moved over the plots to induce drought. LCB moths were then released into the greenhouses to initiate an LCB epidemic. At 99, 113, 127, 141, and 155, 3 m of peanut plants were removed from each row, pods were removed by hand, dried in a drying oven at ca. 45°C, and the pods in each treatment were separated into undamaged, externally scarified, and penetrated classes relative to LCB damage. Pods and kernels from each treatment, replication, and damage class were then analyzed for *Aspergillus flavus* infection and/or aflatoxin contamination. Results showed that insecticide applications had little effect on aflatoxin contamination in peanut kernels under the severe conditions of the tests. In one of two years, Southern Runner had a significantly higher level of aflatoxin contamination than did Florunner. Age of peanuts at harvest and degree of lesser cornstalk borer damage to pods significantly affected aflatoxin contamination in all three years.

Bioassays were conducted to evaluate transgenic peanut plants containing a codon-modified *CryIA(c)* gene from *Bacillus thuringiensis* for activity against the LCB. Bioassay with callus was quite variable. Bioassay of peanut leaflets from regenerated plants was more reliable and showed a high level of toxicity to 7-day-old LCB larvae in T26-1-12, T26-11-53, T26-12-3, and T30-9-140. Only very small feeding scars were noted on these lines and most larvae died. Other lines showed moderate toxicity to LCB larvae as expressed in moderate mortality and reduced larval weight. In moderately resistant lines, slight to moderate feeding was noted on the leaflets, but, in many instances, the larvae did not die within the thumb of the bioassay. Feeding on the untransformed Florunner control and on susceptible transformed lines was extensive and essentially all of the leaf material was consumed in the 7-10 day evaluation period. A significant correlation ($r = 0.7127$, $p = 0.05$) was noted between survival of LCB borer larvae and weights of the survivors. Thus, peanut lines T26-1-12, T26-11-53, T26-12-3, and T30-9-140 contain sufficient insecticidal activity due to the expression of the *CryIA(c)* gene to justify evaluation against other peanut insects.

Incidence of *Aspergillus flavus* Seed Infection and Aflatoxin Contamination in Mississippi Cotton Modules and the Influence of Field Storage

W. E. Batson, Jr. and J. Caceres
Mississippi State University, Mississippi State, MS

In previous surveys of the Mississippi Delta, *Aspergillus flavus* was detected in washings from lint samples from 23 and 47% of fields sampled. However, populations of *A. flavus* were relatively low and modules constructed from selected fields contained very low levels of seed infection and aflatoxin. Aflatoxin was detected in the five modules studied in connection with the survey. In a further effort to determine the extent of *A. flavus* contamination, two one-half pound samples of fuzzy seed were collected from the seed stream of adjacent gin-stands during the ginning of each of 118 modules at the Hope Gin in Thornton, MS. *A. flavus* infected seed were detected in 24 of 118 modules (20%) and ranged from 0.8 to 16.2%. Aflatoxin in low amounts was found in 5 of 24 (21%) of the *A. flavus*-positive modules.

In an effort to increase *A. flavus* seed infection and subsequent aflatoxin contamination, 15 acres of cotton were grown at Mississippi State, MS and selected areas of the field sprayed at boll crack with an *A. flavus* spore suspension. In addition, numerous individual bolls were inoculated and bagged to maintain humidity. Two modules (MSU I and MSU II) were constructed, each with 16 sampling ports. Each module was sampled at module construction and weekly for seven weeks. An additional sample was taken from the exterior of each module beginning at week four for MSU I and week 3 for MSU II. At the time of module construction, aflatoxin was detected from 2 of 16 and 7 of 16 sampling ports from MSU I and MSU II, respectively. The number of sampling ports from which aflatoxin was detected increased with time. Aflatoxin was detected in seed from all 16 sampling ports after two and four weeks of storage for MSU II and MSU I, respectively. Analysis of aflatoxin data indicated a module by time interaction, therefore each module was analyzed separately. Regression analysis of mean aflatoxin concentration (mean of 16 ports) versus time was positive and significant indicating that aflatoxin concentration increased over time in MSU I. The relationship in MSU II was positive but not significant. We also looked at change in aflatoxin concentration over time for each port within each of the modules. There was a significant increase in aflatoxin concentration over time at seven of the 16 ports in MSU I and at one port in MSU II. There were no instances of significant decreases in aflatoxin concentration over time. Aflatoxin was detected in all samples taken from the exterior of modules MSU I and MSU II. Levels were similar to those found within the modules and there was no significant change over time.

Elimination of Aflatoxin in Pistachios and Almonds through Sorting

T. F Schatzki and J. Pan
ARS Western Regional Research Center, Albany CA

Pistachios Additional measurements of aflatoxin distributions p(c) on product streams of processed as well as freshly harvested pistachios has allowed a refinement of this distribution. [Here p(c) is the probability of a nut having a concentration in a range around concentration c.] It is found that p(c) consists of two regions in c: 0.03 ppb (the lower limit of sensitivity) to 1000 ppb and 1000 ppb to 1,000,000 ppb. The lower region exhibits a high probability of infection with p = 0.1 - 0.001 approximately, decreasing rapidly with c. In the upper region p is roughly constant with values around .001 to 0.00001, depending on process stream. The source of aflatoxin at low c is interpreted as arising from "tattered" nuts, where the hull is split in the last two weeks before harvest, while the aflatoxin at high c is believed to arise from early split and insect infection occurring 2-6 weeks before harvest, previously observed by Doster and Michaelides [Aflatoxin Elimination Workshop, October 1994]. An upper limit of aflatoxin level in single nuts of about 1,000,000 ppb is observed in almost all nuts. This upper limit yields two results.

1. For samples smaller than 1/p for high c [i.e. samples smaller than 1000 to 100,000 nuts] sample aflatoxin concentration C will be less than 1,000,000/sample size. This is universally observed.
2. In general, variance(C) = (800,000 nuts/sample size) x mean(C). This latter equation is good to about a factor of two and allows estimate of sample size required for a given mean and variance [or standard deviation].

Measurements have been made of the aflatoxin concentration of small, sorted [high quality] nuts. Nuts of 0.45-0.7 g/nut contain up to 20 ppb of aflatoxin, larger and smaller nuts are substantially aflatoxin free. Preliminary measurements have been made of the development of aflatoxin in wet-stored pistachios. Nuts containing more than 15% moisture [wet basis] show a rapid rise in C, exceeding 100 ppb in 6-8 days. Nuts where moisture < 13% show substantially no aflatoxin development in 40 days. This work is being repeated with more careful control of moisture and closer time intervals.

Almonds The aflatoxin content of 1993 crop almonds has been studied by analyzing the results obtained by a quality control laboratory which services the tree nut industry in California [DFA of California] and by studying the in-house lab reports of some almond processors. Results were broken down by almond quality [in terms of USDA and industry grades] and aflatoxin level. It was found that 18% of diced and 27% of ground almond samples tested positive [>= 1 ppb]; for all remaining grades less than 2% positives were obtained. Only 0.4% of samples exceeded 20 ppb and 1.2% exceeded 4 ppb of total aflatoxin. The overall average amounts to 0.3 ppb, one quarter of that found in pistachios. These results are in good agreement with the results of Schade et al. [Appl. Microbiol. 29:48-53 (1975)].

Publications

T. F. Schatzki, Distribution of Aflatoxin in Pistachios. 1. Lot Distributions. J. Agric. Food Chem. 43:1561-1565 (1994); 2. Distributions in Freshly Harvested Pistachios. J. Agric. Food Chem. 43:1565-1569 (1994)

Relationship of Insect Injury and Cotton Root Rot to Aflatoxin Contamination of Cottonseed in South Texas

T. Isakeit and J.R. Dunlap
Texas A & M University Research and Extension Center, Weslaco

The objective of this study was to evaluate two possible pre-disposing factors to aflatoxin contamination of cottonseed in south Texas: (1) cotton root rot infection; and (2) insect injury. To evaluate aflatoxin pre-disposition as a result of cotton root rot (caused by the fungus Phymatotrichiopsis omnivora), cotton was harvested from fields with large areas showing symptoms of cotton root rot. In each field, cotton was harvested separately from plants showing obvious symptoms of cotton root rot and non-symptomatic plants growing outside of symptomatic areas. In samples from San Patricio county, there was no apparent relationship of aflatoxin concentration in cottonseed from plants with cotton root rot symptoms as compared with apparently healthy plants in the same field. Concentrations of aflatoxin ranged from 0-60 parts per billion (ppb). In some fields, aflatoxin concentration was higher in the cotton root rot areas than in the non-affected areas, but the reverse situation was also seen. All samples from Hidalgo county had concentrations less than 6 ppb. Because we did not see consistent, high concentrations of aflatoxin in association with diseased plants, we conclude that cotton root rot is not an important predisposition factor for aflatoxin contamination. To determine if insect injury of the boll prior to maturity was an important factor in aflatoxin contamination, cotton was sampled from modules and gin carts at seven gin yards in San Patricio county from August 25 to September 14, 1995. Locks were examined for the presence of bright green yellow fluorescent (BGYF) lint, which indicates infection prior to boll maturity, as a consequence of insect injury. Seed associated closely with BGYF lint was segregated from non-BGYF lint and analyzed separately for aflatoxin. BGYF lint was found in 23 out of 29 (79%) module and gin cart samples from San Patricio county. Seed associated with BGYF lint comprised 0.009 - 0.31% of the samples. High aflatoxin concentrations (i.e. greater than 15 ppb) were found in 76% of non-BGYF samples and in 53% of BGYF samples. However, BGYF seed tended to have higher concentrations of aflatoxin than non-BGYF seed. While non-BGYF seed samples had a range of 0-5,000 ppb aflatoxin, BGYF samples had a range of 0-218,750 ppb. A very small percentage of highly-contaminated BGYF seeds can cause the rest of the non-BGYF sample to exceed the threshold of allowable aflatoxin (15 ppb). For example, in one sample, we found three BGYF seeds with a concentration of 218,750 ppb. The presence of these three seeds, 0.009% of the total, will cause 1½ pounds of non-BGYF cottonseed to exceed the allowable aflatoxin limit. Important boll insects in San Patricio county in 1995 included the boll weevil, the tobacco budworm and the tobacco bollworm. In conclusion, our study showed that insect injury can be a significant predisposition factor for aflatoxin contamination in south Texas.

Hydrogen Peroxide Treatment of Pistachios to Reduce Aflatoxins and Microbes

G.F. Simmons, J.L. Smilanick, and D.A. Margosan
USDA,ARS Horticultural Crops Research Laboratory Fresno CA

P.C. Bolin, J.M. Hurley, and T. Jones
Dried Fruit Association Laboratory of California Fresno CA

An aflatoxin producing *Aspergillus flavus* strain (KAC 715) isolated from pistachios was obtained from M.A. Doster and T.J. Michailides (U.C. Davis) for this study. The Dried Fruit Association Laboratory (DFA), at which all aflatoxin analytical work was conducted, confirmed the strain capable of producing aflatoxin concentrations greater than 2000 ppb within 4 days of nut meat inoculation.

A. flavus spores were collected from 2-week old sporulating fungal cultures, diluted to a concentration of 1 X 10^6 spores/ml, and sprayed on hydrated pistachio nut meats. Inoculated nut meats were incubated in 30 liter plastic boxes on screens at 30 ^{0}C in a walk-in growth room. Inoculated containers of nut meats were removed each day for 7 days and dried at 60 ^{0}C for 24 hours. The 3rd and 7th day nut meats were analyzed.

Replicated, dried *A. flavus* inoculated pistachio nut meats from the 3rd and 7th days were divided into 250 gram samples for treatments. The 1st sample was an untreated control, the 2nd sample was washed 30 minutes in alkaline water (pH 10), and the 3rd sample was washed 30 minutes in 10 % alkaline (pH 10) hydrogen peroxide. After washing, the samples were air dried for 2 days.

The DFA laboratory ground the dried nut meats in Waring blender cups, extracted the samples with 60 % methanol, filtered, diluted, and cleaned the samples with Vicam AflaTest™ affinity columns. The aflatoxin concentrations were determined against standards on a HPLC using fluorescence detection.

The mean aflatoxin concentrations in nut meats declined after either the alkaline water or peroxide treatments in comparison to the untreated controls for both the 3rd and 7th day. The mean ppb from 3 replicates for the 3rd day aflatoxin concentrations were 3776, 2711, and 2548 for untreated, alkaline water washed, and alkaline peroxide washed nut meats, respectively. The mean ppb from 3 replicates for the 7th day aflatoxin concentrations were 32917, 19353, and 21417 for untreated, alkaline water washed, and alkaline peroxide washed nut meats, respectively. Although the means suggest a reduction in aflatoxin from the water and peroxide treatments, it was not statistically significant. The increase in aflatoxin concentration from the 3rd to the 7th day was statistically significant (P=0.001.

The rapid increase in pistachio aflatoxin concentration after inoculation shows the nut meats to be a good substrate for aflatoxin production. The rapid increase in aflatoxin concentration suggests risk of high levels of aflatoxin associated with storing wet pistachio nut meats or having nut meats becoming accidently hydrated while in storage. However, the natural spore inoculation level would not be as high as the amount used in this experiment. The process of washing the pistachio nut meats may have reduced the aflatoxin concentrations by removing a portion of the dried mycelia mat which covered the nut meats.

An American Sterilizer VHP™ 1000 was used to generate 3mg/liter volume hydrogen peroxide vapor into a stream of dry air. One year old pistachio nut meats were exposed in a column to 5, 10, 15, 20, 30, and 60 minutes hydrogen peroxide vapor. Afterward replicated 50 gram samples of treated and untreated pistachio nut meats were washed for 2 minutes with a phosphate buffer pH 7.2. Serial dilutions were made of the buffered wash water. The serial dilutions were spread plated on aerobic plate count (PCA) and dichloran rose bengal (DRBC) agar in duplicate petri dishes. The PCA and DRBC containing petri dishes were incubated at 36 °C for 2 days and 25 °C for 5 days, respectively. The colony counts were made under magnification after the incubation periods and the means for 3 replicates were calculated.

PCA agar excludes the growth of most molds and those bacteria unable to grow at 36 °C. The colony forming units per gram (cfu/g) on untreated pistachio nut meats plated on PCA were 17591. The cfu/g on treated nut meats were 545, 3132, 580, 41, 5, and 160 after vapor peroxide exposure times of 5, 10, 15, 20, 30, and 60 minutes, respectively.

DRBC agar selects for the growth of molds over bacteria and suppresses their spreading habit of growth. The cfu/g on untreated pistachio nut meats plated on DRBC were 6150. The cfu/g on treated nut meats were 75, 63, 543, 6, 92, and 25 after vapor peroxide exposure times of 5, 10, 15, 20, 30, and 60 minutes, respectively.

Vapor hydrogen peroxide substantially reduced the microbe counts on treated pistachio nut meats as measured on both PCA and DRBC agar. No differences were found between 5-60 minutes vapor hydrogen peroxide exposure times. Vapor hydrogen peroxide appears to be an alternative to the presently utilized propylene oxide for the reduction of microbes on pistachio nut meats.

The use of hydrogen peroxide, a Generally Recognized As Safe Substance under present Food and Drug Administration regulations, would require at least a self affirmation of GRAS. Peter Barton Hutt, former chief counsel for the Food and Drug Administration, made a presentation on this subject at the Institute of Food Technologists annual meeting at Anaheim, California June 5, 1995.

MACHINE RECOGNITION OF INSECT DAMAGE IN X-RAY IMAGES OF TREE NUTS.

PAMELA M. KEAGY[1], BAHRAM PARVIN[2] AND THOMAS F. SCHATZKI[1]
[1]USDA-ARS Western Regional Research Center, 800 Buchanan St., Albany, CA 94710
[2]Lawrence Berkeley Laboratory, ICSD-ITG MS 50B-2239, 1 Cyclotron Rd., Berkeley, CA 94720

Insect infestation increases the probability of aflatoxin contamination of pistachio nuts. Manual inspection is used to remove nuts with external evidence of insect infestation just prior to roasting. An automated inspection technique is needed to improve completeness and consistency of insect removal, remove infested nuts which don't show external evidence of infestation, and reduce costs of manual inspection. X-ray film images reveal evidence of insect infestation which can be readily recognized by a trained observer. The continuing objective of this work is to develop an X-ray based automated inspection method for insect damage in pistachio nuts.

Last year we presented and published recognition data from two image processing approaches which indicated that 85% of insect infested nuts could be identified in small normal pistachios with 5.5% of the control nuts misclassified. If only 0.5% misclassified controls were allowed, then insect detection dropped to 50%. In order to develop and test algorithms with errors rates of 1% and below a large image data set is required. We have obtained samples and prepared an image data base of approximately 9000 infested and control nuts of various sizes. The data base consists of film and realtime linescan images accompanied by dissection information confirming the type of damage. The images are being used to refine and further evaluate current algorithms. In addition, the images are being made available to collaborators for screening and development of new recognition approaches.

Pinhole damage due to attack by small worms occurs in almonds and is nearly impossible to detect in natural almonds (unblanched). It is possible that similar to other types of insect damage, pinhole damage carries with it potential for mold introduction and toxin production. We have found that pinhole damage in almonds can be revealed in film x-ray images. An image database consisting of 522 almonds containing pinholes and 1565 control almonds has been created. This image set is being used to develop recognition algorithms. Preliminary results with images scanned from film indicate that we can easily detect 65% of the pinhole almonds with 4% of the controls being misclassified. Other algorithms will detect approximately 90% of the infested nuts but misclassification of the controls increases to 10 to 15%. We are currently working on subroutines which will eliminate the primary cause of misclassified controls, cracks between the germ and cotyledons of the nut.

Publications:

Keagy, P. M., Parvin, B. and Schatzki, T. F. "Machine recognition of navel orange worm damage in x-ray images of pistachio nuts" in Optics in Agriculture, SPIE Proc. 2345, 1994. Paper presented Nov. 3 1994, SPIE meeting, Boston, Ma.

Keagy, P.M., Parvin, B. and Schatzki, T.F. Machine recognition of navel orange worm damage in X-ray images of pistachio nuts. Paper Accepted Food Science and Technology.

Sim, A., Parvin, B. and Keagy, P. Machine Vision Inspection of Insect Infested Pistachio Nuts from X-ray Images. Proceedings Vision Interface '95, Canadian Image Processing and Pattern Recognition Society, p17-22, May 16-19, 1995.

Sim, A., Parvin, B. and Keagy, P. Invariant representation and hierarchical network for inspection of nuts from X-ray images. Abstract IEEE International Conference on Neural Networks. Nov. 27 to Dec. 1, 1995, Perth, Western Australia.

Machine vision system for real time detection of stained pistachio nuts

Tom Pearson, USDA-ARS, WRRC, Albany, CA

The pistachio industry currently utilizes a variety of methods and equipment to remove stained nuts. Bi-chromatic color sorters are used to remove badly stained nuts. These devices measure the "average color" of the entire nut surface. However, many unstained nuts have wide shell openings, exposing more of the dark kernel, which causes the average nut color to have a more reddish hue. As a result, an unstained nut with a wide shell split might be rejected by the bi-chromatic color sorter. Approximately 50% of the nuts rejected by the bi-chromatic color sorters are not stained. Conversely, some nuts accepted by the bi-chromatic color sorter have small stains with insufficient area to affect the average nut color. The machine vision system developed has the computational power and image resolution to distinguish unstained nuts from slightly stained and heavily stained nuts, regardless of kernel exposure through the shell split.

The machine vision system was built by modifying a monochrome sorter (ESM, Microscan II, Houston, TX). This device uses three line scan cameras (256 pixels/line, 200 KHZ pixel rate) to scan the entire perimeter of the nut. To distinguish a stained pistachio nut from an unstained nut, the output of each camera is diverted to a digital signal processing (DSP) board hosted in a 486-33 personal computer (PC). In the video signal, unstained portions of the nuts have low intensity and low gradient (change in intensity from pixel to pixel) values. Pixels representing stained portions of the nut have higher intensity and gradient values. The DSP counts the number of pixels with both a high intensity and gradient, number of pixels with both a low intensity and gradient, and also computes the number of regions in the video signal with high intensities and gradient values. This third parameter indicates the number of stained and unstained regions on the nut. These three parameters are combined on the PC after the nut passes out of view from the camera. Discriminate analysis was used to develop discriminate functions to determine if a nut is stained or unstained. The PC uses the discriminate functions to compute the probability of the nut being stained. Depending on this probability, the PC can activate an air nozzle to reject the nut.

The system was developed to remove stained nuts from process streams that may contain high levels of aflatoxin. It is believed that the stained nuts, particularly nuts with stains adjacent to the suture, may be more likely to be contaminated with aflatoxin. However, more research is required to assess the ability of the machine to remove aflatoxin contaminated nuts.

The machine vision system may have other quality control applications such as recovering unstained nuts from the reject streams of the bi-chromatic color sorters. For this type of application, the machine vision system can recover approximately 90% of the unstained nuts from the entire bi-chromatic color sorter reject stream while also accepting about 10% stained nuts. The maximum throughput rate of the machine vision system is 40 nuts/s (500 lb/hr) and the cost is estimated to be about $15,000. If recovered unstained nuts could be sold at a rate of $0.15/lb higher than if they remained in the reject stream, the machine vision system would have a payback time of only 8 weeks based on a 50 hour week.

The machine may have applications with other pistachio process streams or commodities such as string beans. The prototype and developmental work on this system is complete and a patent has been applied for.

Publications:

Pearson, T. "Machine Vision System for Automated Detection of Stained Pistachio Nuts." in Optics in Agriculture, SPIE Proc. 2345:95-103 (1994)

Pearson, T. "Machine Vision System for Automated Detection of Stained Pistachio Nuts." Paper accepted by Food Science and Technology.

Drought Stress-Induced Loss of Kernel Integrity and Its Contribution to Preharvest Aflatoxin in South Texas Corn. **G.N. Odvody, Texas A&M Research & Extension Center, Corpus Christi, TX.**

A preharvest loss of kernel integrity called silk cut is an important recurring problem of many commercial corn, maize (Zea mays L.) hybrids exposed to late season drought stress in South Texas. Silk cut is defined as the preharvest occurrence of one or more lateral splits in the kernel pericarp that expose internal kernel tissues and the embryo to either pre- or postharvest (storage) attack by fungi and insects. Silk cut can occur as multiple lateral splits anywhere on the seed surface but its primary and most common occurrence is as single, lateral splits at the kernel edge on either or both sides of the embryo. Nonlateral (longitudinal) splitting and streaking of the kernel pericarp is another loss of kernel integrity occurring independent of and simultaneously with silk cut. Silk cut can be initiated at kernel moistures as high as 50% but is more commonly initiated and observed at kernel moistures of 28% and lower. Incidence of silk cut is highly variable between ears of adjacent plants even on susceptible hybrids exposed to stress environments favorable to silk cut. Incidence of silk cut is higher in kernels at the top of the ear, especially if whole ear incidence is low. *Fusarium moniliforme,* mating population 'A', is the predominant, visible colonizer of silk cut kernels, especially at higher kernel moistures, but *Aspergillus flavus* colonization is often sufficient at all moistures to affect aflatoxin content. Silk cut incidence and severity is associated with increased aflatoxin content and its own variability further contributes to aflatoxin variability in the field. Hybrids differ in vulnerability to silk cut but incidence is variable across years, sites, and stress environments even on the most vulnerable hybrids. Hybrid vulnerability to silk cut is associated with loose husks and open ear tips and resistance is associated with tight, closed husks. Under late season drought stress, open husks may allow premature drying and hardening of kernel surfaces which lead to loss of kernel integrity including silk cut.

Poster Abstract for Aflatoxin Elimination Workshop
Atlanta, GA, 1995

PROGRESS TOWARDS NEW SEMIOCHEMICAL ATTRACTANTS OF LEPIDOPTEROUS PESTS ASSOCIATED WITH *ASPERGILLUS* INFECTION OF TREE NUTS

Light, D. M., Flath, F. A., Merrill, G. B., Teranishi, R., and Campbell, B. C.
Western Regional Research Center, USDA-ARS, Albany, CA 94710

Aflatoxins are a group of natural products synthesized by certain strains of species of fungi in the genus *Aspergillus*. Aflatoxins are associated with human hepatocellular carcinomas and teratogenic birth defects. Occurrence of aflatoxins in tree nuts (*e. g.*, almonds, walnuts and pistachios) is a growing concern to food safety in the United States. Insect probing and feeding damage to nut husks, hulls, shells and kernels creates pathways for infection by *Aspergillus* fungi which may lead to aflatoxin contamination.

There are three major insects pests of tree nuts in California. These include navel orangeworm (NOW), peach twig borer (PTB) and codling moth (CM). NOW infests kernels (nutmeats) of almonds, walnuts and pistachios. PTB infests the meristem leaf shoots, husks and kernels of almonds. An additional pest of walnuts, CM, infests kernels. Disruption of normal mating, oviposition, feeding and dispersal behaviors of these insect pests will reduce aflatoxin contamination of tree nuts. The behaviors of these insects are mediated by natural products termed semiochemicals (*viz.*, pheromones, host-plant volatiles, *etc.*). Dependency of these insects on semiochemicals allows for a unique means to monitor population densities, times of emergence, and dispersal and also provides a vulnerability that can be exploited for disrupting normal biology of these pests. Effectiveness of pheromones can vary, however, between genetically divergent moth populations. Combined with population genetic studies, biorational programs for control of these insects pests can be optimized.

Female moths perform all sexual activities on their host-plant. A combination of female produced pheromone(s) and host-plant volatiles are emitted during the process of mate "calling". These insects have adapted extremely sensitive capabilities to detect and discriminate such natural compounds. Control or monitoring systems that incorporate appropriate mixtures of insect pheromone and host-plant volatiles show improved activity at a synergistic level. Field bioassays of blends of pheromone + host-plant volatiles can enhance trapping of PTB and CM. Plant volatiles that enhance capture of commercially available pheromones of either PTB or CM include, methyl octanoate, limonene, and (E,E)-α-farnasene and a blend of six-carbon aldehydes and alcohols. Gas chromatographic-mass spectroscopic analysis of head space trappings of tree nut volatiles identified further candidate volatiles to be tested as synergists to pheromonally-based baits. Further research will isolate and identify pheromone/host-plant volatile blends that will improve control of these tree nut pests in an environmentally benign systems management approach.

These insect pests have a variety of host-plants, in some cases genetically distant (*e.g.*, walnuts and apples for CM). Further research will determine to what extent blends of volatiles from non-tree nut hosts may enhance the activity of pheromones in tree nut orchards. Based on initial research, host-plant volatiles, some from alternative host-plants (*e.g.*, peaches), enhance attractancy of pheromone traps of nut-pest moth species. These experiments, combined with further genetic identification of moth population diversity, are encouraging signs towards future development of improved and highly potent pheromone monitoring and control systems.

IPM of Aflatoxin in the Corn Belt - FY 1995 Results.

P.F. Dowd, G.A. Bennett, J.L. Richard (MTX Research Unit, USDA-ARS, NCAUR), R.J. Bartelt, R.W. Behle, M.R. McGuire, F.E. Vega (BC Research Unit, USDA-ARS, NCAUR), G.E. Scott (USDA-ARS, CSRL), T. Brandhorst and W. Kenealy (J. Whittier Biologics), J.S. Ferguson, G. Warren (CIBA Seeds), D. Kenney (Gustafson Corp.), L.M. Lagrimini (Ohio State University).

Biological Control

Males of the *Hexamermis* nematode parasite of the dusky sap beetle (a vector of mycotoxigenic fungi to corn) were found to occur in the antique sap beetle and lesser flattened bark sap beetle at levels of 40% of the total. Introductions to new sites should include all three species to help insure sufficient males. Male nematodes occurred earlier in the spring than females.

The Kodiak strain of *B. subtilis* was successfully encapsulated in corn flour and survived 1 year in the matrix when stored on the shelf. When applied as granules to corn at pollination, the material signficantly reduced mold colonization of pollen and anthers in leaf axils. The bacteria could be recovered from axil material, European corn borer frass, corn kernels, and sap beetles found on damaged corn and collected from distant traps. Incidence of mold on damaged ears was reduced compared to distant, untreated areas in the same field.

A RAPD PCR method of identifying strains of the insect pathogen *Beauveria bassiana* was developed. It is being used to track a strain released from an autoinoculative device coupled with a sap beetle trap as part of a study aimed toward increasing mortality of overwintering sap beetles.

A simple to prepare, heat and dryness tolerant fermenting gel attractant for sap beetles was prepared that remained highly attractive during hot, dry periods of the summer over a 3 week period. It is hoped this will be more readily accepted than definded chemical attractants when highly precise release rates are not necessary.

Resistance mechanisms

The number of peroxidase isozymes in aflatoxin-resistant inbreds was generally greater than in aflatoxin-susceptible inbreds, but exceptions did occurr. Apparently additional factors that may interact with peroxidases need to be quantitated to get a clear picture of the importance of peroxidases in aflatoxin susceptibilty (some susceptible varieties appear deficient in key isozymes). Both *B. subtilis* and *A. flavus* induced peroxidase isozymes and isozyme activity in certain corn hybrids compared to sham controls. There was also some evidence of isozyme induction remote from the inoculation site.

Transgenic corn expressing a BT crystal protein in all plant parts (including kernels and silks) had greatly reduced mold incidence compared to wild type ears when all ears were first inoculated with newly hatched European corn borers, and one week later with a mixed spore suspension of mycotoxigenic *Fusarium proliferatum* and *Aspergillus flavus*. European corn borer damage was absent from the transgenic plant ears, but greater than 50% in wild type plants. Poor fill, which was apparently due to death of pollen in bagged ears that occurred with these and other varieties being pollinated at the same time due to

extremely hot temperatures, did not provide enough kernels for meaningful toxin determinations.

Leaf resistance to corn earworms in rosette plants of transgenic tobacco varieties that over- and under-produce a tobacco anionic peroxidase was dependant on leaf age and prior damage. Overproducers had intermediate leaves more resistant than wild type. Underproducers that had prior damage had less resistance to caterpillars than wild type plants that had prior damage.

The fungal protein restrictocin apparently serves a defensive role in *Aspergillus resitrictus*, as structures containing high levels of the protein were avoided by the Freeman sap beetle. At 1000 ppm in diets, either mortality or avoidance was seen with adults and larvae of the Freeman sap beetle, fall armyworm caterpillars, and maize weevil adults.

Insecticide Encapsulation

Corn flour encapsulated malathion was tested in small plots using commercial application techniques (highboy with ear zone delivery tubes). The two highest granular rates (1 lb, 0.1 lb a.i./A) provided significant control of European corn borers at milk stage, but the lowest rate of granules (0.01 lb/A a.i.) and 5 sprays of malathion at 1 lb a.i./A (conventional application and formulation) did not. Based on numbers of dead insects found, control of beetles feeding in leaf axils (sap beetle vectors and a newly discovered potential scarab beetle vector) was greater overall with the highest rate of granules, followed by the intermediate rate, then the lowest rate and the 5 sprays of malathion (which were approximately equivalent). Approximately 4 times as many dead lady beetles were found in the plots treated with the 5 sprays as in the untreated or granule treated plots (which were approximately equivalent). Due to harvest by the cooperator prior to evaluation, no mold determinations could be performed.

Coverage small plot studies using whirly-bird spreader application indicated insect and indirect mold control at twice the coverage with a total of 0.05 lb/A a.i. was not signficantly different from 0.1 lb/A a.i. European corn borers in ears were significantly controlled for ca. 10 days by the granule treatments overall (two generations attacked ears at this site). The numbers of sap beetles found dead in granule treated plots were from 30-40X the number found in control plots. At harvest both corn varieties tested that were treated with granules had the incidence of mold significantly reduced by about 3-fold compare to untreated plots (from ca. 30% to ca. 10% and from ca. 15% to ca. 5% overall).

Relevant Publications

Dowd, P.F. 1994. Enhanced maize (*Zea mays* L.) pericarp browning: Associations with insect resistance and involvement of oxidizing enzymes. J. Chem. Ecol. 20: 2497-2523.

Dowd, P.F. 1995. Sap beetles and mycotoxins in maize. Food. Add. Contam. 12: 497-508.

Dowd, P.F. and Nelsen, T.C. 1994. Seasonal variation of sap beetle (Coleoptera: Nitidulidae) populations in central Illinois cornfield-oak woodland habitat and potential influence of weather patterns. Environ. Entomol. 23: 1215-1223.

Dowd, P.F. and R.A. Norton. 1995. Browning-associated mechanisms of resistance to insects in corn callus tissue. J. Chem. Ecol. 21: 583-600.

PREDICTION OF AFLATOXIN CONTAMINATION IN PREHARVEST PEANUTS

G. Hoogenboom, R.S. Parmar, R.W. McClendon, P.D. Blankenship, R.J. Cole, J.W. Dorner, and C. Pinto.

Prevention and elimination of aflatoxin contamination of preharvest peanuts requires identifying the factors involved in the contamination process and evaluating the effects of those factors on contamination levels. The objectives of our study were to determine the environmental variables that affect this contamination process and develop a model to predict aflatoxin contamination levels. An artificial neural network was identified as an appropriate technique because of its ability to assign importance levels to the input parameters and map inputs into outputs when the relationship between them is not known. Seven years of preharvest peanut aflatoxin data were used to train and test neural networks. The data were randomly divided into a training set and a test set. Neural networks were developed using various network architectures and combinations of environmental variables as network inputs. The combinations of inputs were obtained from four environmental variables: soil temperature, drought, crop age, and accumulated heat units. The accumulated heat units were computed based on threshold soil temperatures ranging form 23 to 29°C. The back-propagation algorithm with a logistic activation of hidden and output nodes and three layers of nodes was used. The most accurate predictions were achieved when a threshold soil temperature of 25°C was used to compute accumulated heat units and all four environmental variables were used as inputs in a network with eight hidden nodes. The R^2-values for the training and the test sets were 0.9250 and 0.9522, respectively. In general, the environmental variables and threshold soil temperature used to compute accumulated heat units had a major effect on prediction accuracy while number of hidden nodes had a small effect. A comparison with a traditional statistical approach, using step-wise linear regression, showed that the neural network models were more accurate with their predictions than the statistical models. It can be concluded from this study that neural networks can be used to develop a model that predicts aflatoxin contamination in preharvest peanuts as a function of environmental variables and management scenarios.

The authors are: Gerrit Hoogenboom, Associate Professor, Biological and Agricultural Engineering Department, University of Georgia, Griffin; Rajbir S. Parmar, Research Engineer, Ronald W. McClendon, Professor, Clyde Pinto, Graduate Research Assistant, Biological and Agricultura Engineering Department, University of Georgia, Athens; P.D. Blankenship, Agricultural Engineer, R.J. Cole, Microbiologist, and J.W. Dorner, Microbiologist, USDA-ARS, National Peanut Research Laboratory, Dawson, Georgia

8th Annual Aflatoxin Elimination Workshop

Registration List

October 23 & 24, 1995

Doubletree Hotel, Atlanta, Georgia

William Anderson
University of Georgia
AgraTech Seed Research
P. O. Box 644
Ashburn, GA 31714
(912) 567-3438
(912) 567-2043 FAX

Dean Barry
USDA/ARS
243 Ag Energy Bldg
Columbia, MO 65211
(314)882-1116
(314)875-5359FAX

W.E. Batson
USDA
Mississippi State Univeristy
P.O. Box 9655
Mississippi State, MS 39762
(601)325-2585
(601)325-8955FAX

Antoinette A. Betschart
USDA, ARS, PWA-Western Regional Research Center
800 Buchanan Street
Albany, CA 94710
(510) 559-5600
(510) 559-5963 FAX

Deepak Bhatnagar
USDA-ARS-Southern Regional Center
1100 Robert E. Lee Blvd.
New Orleans, LA 70124
(504) 286-4388
(504) 286-4419 FAX

Paul C. Bolin
DFA of California
1855 So. Van Ness Avenue
Fresno, CA 93721
(209) 233-0604
(209) 233-9819 FAX

Kira L. Bowen
209 Life Sciences
Auburn University
Auburn, AL 36849
(205)884-1953
(205)884-1947FAX

Kyd Brenner
Corn Refiners Association
1701 Pennsylvannia Avenue, NW
Suite 950
Washington, DC 20006
(202) 331-1634
(202) 331-2054 FAX

Ray A. Bressan
Purdue University
1165 Horticulture Building
West Lafayette, IN 47907
(317) 494-1366
(317) 494- 0391 FAX

Robert Brown
USDA-ARS-SRRC
1100 Robert E. Lee Blvd.
New Orleans, LA 70124
(504) 286-4359
(504) 286-4419 FAX

Lori Bunch
J. Leek Associates
P. O. Box 50395
Albany, GA 31703
(912) 889-8293
(912) 888-1166 FAX

Gloria Burow
Dept. Of Plant Pathology & Mirco
Texas A&M University
College Station, TX 77843

Bruce Campbell
USDA,ARS, WRRC
800 Buchanan Street
Albany, CA 94710
(510) 559-5831
(510) 559-5777 FAX

Jeff Cary
USDA/ARS SRRC
1100 Robert E. Lee Blvd.
New Orleans, LA 70124
(504)286-4264
(504)286-4419FAX

Chris Chatzidakis
The Procter & Gamble Company
Miami Valley Laboratories
P. O. Box 538707
Cincinnati, OH 45253
(513) 627-1849
(513) 627-0761 FAX

Caryl Chlan
Biology Dept.
University of S.W. Louisiana
Lafayette, LA 70504
(318)482-5916
(318)482-5660

Fun S. Chu
University of Wisconsin
1925 Willow Drive
Madison, WI 53706
(608) 263-6932
(608) 263-1114 FAX

Thomas E. Cleveland
USDA/ARS, Southern Regional Center
P. O. Box 19687
New Orleans, LA 70179
(504) 286-4387
(504) 286-4419 FAX

Dick Cole
USDA/ARS/NPRL
1011 Forrester Drive, SE
Dawson, GA 31742
(912) 995-7404
(912) 995-7416 FAX

Peter J. Cotty
USDA-ARS-SRRC
P. O. Box 19687
New Orleans, LA 70179
(504) 286-4391
(504) 286-4419 FAX

Kimberly J. Cutchins
National Peanut Council
1500 King Street, Suite 301
Alexandria, VA 22314
(703) 838-9500
(703) 838-9089 FAX

Abhaya Dandekar
Department of Pomology
University of California
Davis, CA 95618
(916)752-7784
(916)752-8502FAX

W. Diener
411 Summertree Drive
Auburn, AL 36830
(205)887-5606
(205)844-1947FAX

WM. Dolezal
Pioneer Hi-Bred
7300 NW 62nd St.
Johnston, IA 50131

Joe Dorner
USDA/ARS/NPRL
1011 Forrester Drive, SE
Dawson, GA 31742
(912) 995-7408
(912) 995-7416 FAX

Mark Doster
Kearney Agricultural Center
9240 South Riverbend Avenue
Parlier, CA 93648
(209) 891-2500
(209) 891-2593 FAX

Pat Dowd
USDA,ARS
1815 N. University Street
Peoria, IL 61604
(309) 681-6242
(309) 681-6686 FAX

James R. Dunlap
TAES
2415 E. Highway 83
Weslaco, TX 78504
(210) 696-5623
(210) 968-0641 FAX

Jon Duvick
Pioneer Hi-Bred Intl Inc.
P.O. Box 1004
Johnston, IA 50131
(515)270-3176
(515)253-2149FAX

Eugene J. Faron III
American Maize-Products Co.
1100 Indianapolis Blvd.
Hammond, IN 43620
(219) 569-2000 ext 209
(219) 473-6606 FAX

Lance A. Forster, Jr.
National Cottonseed Products Assn.
P. O. Box 172267
Memphis, TN 38187
(901) 682-0800
(901) 682-2856 FAX

Baozhu Guo
Department of Plant Pathology
302 Life Sciences Building
Louisana State University
Baton Rouge, LA 70803
(504) 388-1371
(504) 388-1415 FAX

Tom Gradziel
U C Davis
Pomology Department
Davis, Ca 95616
(916)752-1575
(916)752-8502FAX

Kenneth C. Hancock
M&M/Mars
Albany Georgia Plant
Oakridge Drive
Albany, GA 31706-3289
(912) 883-4000 ext. 412

Gerritt Hoggenboom
Department of Biological & Agricultural Engineering
Georgia Station
Griffin, GA 30223
(770) 228-7216
(770) 228-7218 FAX

Corley Holbrook
USDA/ARS
Coastal Plain Experiment Station
P.O. Box 748
Tifton, GA 31793-0748
(912)386-3176
(912)386-7785FAX

Bruce W. Horn
USDA/ARS/NPRL
1011 Forrester Drive, SE
Dawson, GA 31742
(912) 995-7410
(912) 995-7416 FAX

Sylvia Hua
USDA-ARS, Western Regional Research Center
800 Buchanan Street
Albany, CA 94710
(510) 559-5905
(510) 559-5777 FAX

Zheng-Yu Huang
North Carolina State University
P.O. Box 7616
Raleigh, NC 27695

Tom Isakeit
Texas A&M University
2401 East Highway 83
Weslaco, TX 75896
(210) 968-5581
(210) 969-5639 FAX

Lynn Jones
National Cottonseed Products Assn.
P. O. Box 172267
Memphis, TN 38187
(901) 682-0800
(901) 682-2856 FAX

John Jordan
USDA
1100 Robert E Lee Blvd.
New Orleans, La 70124

Nancy Keller
Dept of Plant Pathology & Mircobiology
Texas A&M University
College Station, TX 77843
(409)845-7311
(409)845-6483FAX

Douglas Light
USDA,ARS, WRRC
800 Buchanan Street
Albany, CA 94710
(510) 559-5831
(510) 559-5777 FAX

John Linz
Michigan State University
234 B GM Trout Food Science Bldg.
E. Lansing, MI 48824
(517) 353-9624
(517) 353-8963 FAX

Robert E. Lynch
USDA-ARS
IBPMRL, P. O. Box 748
Tifton, GA 31793
(912) 387-2375
(912) 387-2321 FAX

Noreen Mahoney
USDA
800 Buchanan Street
Albany, CA 94710
(510) 559-5971
(510) 559-5777 FAX

Charlie A. Martinson
Iowa State University
329 Bessey Hall
Ames, IA 50011
(515) 294-1062
(515) 294-9420 FAX

Mike Matherson
University of Arizona
6425 W. Eighth Street
Yuma, AZ 85364
(520) 726-0458
(520) 726-1363 FAX

Denis McGee
Iowa State University
160 Seed Science Center
Ames, IA 50010
(515)294-7560

Gale McGranahan
University of California
Pomology Department
Davis, CA 95616
(916)752-0113
(916)752-8502FAX

Mary Lou Mendum
University of California
Department of Pomology
Davis, CA 95616
(916) 758-7542

Filmore Meredith
Richard Russell Res. Center
USDA/ARS
P.O. Box 5677
Athens, GA 30604

Themis J. Michailides
Berkeley/Kearney Agricultural Center
9240 S. Riverbend Avenue
Parlier, CA 93648
(209)891-2500
(209)891-2593FAX

Olanya Ocen Modesto
Iowa State University
164 Seed Science Center
Ames, IA 50010
(515) 294-5311
(515) 294-9420 FAX

Merritt R. Nelson
University of Arizona
Department of Plant Pathology
Tucson, AZ 85721
(520) 621-7160
(520) 621-9290 FAX

Robert A. Norton
USDA, ARS
1815 N. University Street
Peoria, IL 61604
(309) 681-6251
(309) 681-6686 FAX

Gary L. Obenauf
Agricultural Research Consulting
3425 N. First Street, #101
Fresno, CA 93726
(209) 244-4710
(209) 224-2610 FAX

Peggy Ozias-Akins
Coastal Plain Experiment Station
Horticulture Department
P.O. Box 748
Tifton, GA 31793
(912) 386-3902
(912) 386-9956 FAX

Gary Odvody
Texas A&M Univesity
TAES RT 2, Box 589
Corpus Christi, TX 78410

Douglas Park
Louisiana State University
Department of Food Science
Baton Rouge, LA 70893
(602)621-5107
(602)621-9446FAX

Gary Payne
North Carolina State Univeristy
P.O. Box 7616
Dept. Plant Pathology
Raleigh NC 27695-7616
(919)515-6994
(919)515-7716FAX

Jim Perkins
DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-9524
(815) 758-4106 FAX

Cesar Reyes
P.O Box 70
Progresso, TX 75938

Jane Robens
USDA Agricultural Research Service
Room 210, Bldg. 005, BARC-West
Beltsville, MD 20705
(301) 504-5381
(301) 504-5467 FAX

Torbert Rocheford
University of Illinois
Turner Hall
Corp Sciences Dept
Urbana, IL 61801
(217)333-9643

IdaYates Russell
Res. Center/ARS-USDA
P.O. Box 5677
Athens, GA 30605

John S. Russin
Louisiana State University
Department of Plant Pathology
Baton Rouge, LA 70803
(504) 388-4880
(504) 388-1415 FAX

Bob Sacher
Hunt-Wesson, Inc.
1701 West Valencia Drive
Fullerton, CA 92633
(714)680-2811
(714)449-5166FAX

T. Shatzki
USDA-ARS-WRRC
800 Buchanan Street
Albany, CA 94710
(510) 559-5672
(510) 559-5777 FAX

Mehboob B. Sheik
Florida Agricultural and Mechanical University
Division of Agricultural Sciences
1500 Martin Luther King Blvd.
Tallahassee, FL 32307
(904) 561-2218
(904) 561-2221 FAX

Richard Shelby
Auburn University
Plant Pathology
209 Life Science
Auburn, AL 36849

Gilbert Simmons
USDA, ARS
2021 S. Peach Avenue
Fresno, CA 93727
(209) 453-3172
(209) 453-3088 FAX

David R.Smith
Dekalb Genetics Corporation
3100 Sycamore Rd
Dekalb, IL 60105
(815)758-9142
(815)758-4106FAX

Doug Smyth
Planters Company
200 DeForest Avenue
East Hanover, NJ
(201) 503-4877
(201) 503-3929 FAX

Victor S. Sobolev
USDA/ARS/NPRL
1011 Forrester Drive, SE
Dawson, GA 31742
(912) 995-7446
(912) 995-7416 FAX

Rick Sousa
Almond Board of California
1104 12th Street
Modesto, CA 95354
(209) 549-8262 ext. 28
(209) 549-8267 FAX

James E.Thornton
Demeter Biotechnologies Ltd
Brightleaf Square-Suite 19D
905 West Main Street
Durham, NC 27701
(919) 762-2826
(919) 682-8340 FAX

Lois H.Tiffany
Iowa State University
Dept. of Botony
Ames, IA 50011
(515) 294-3523
(515) 294-1337 FAX

Frances Trail
Michigan State University
234 Food Sciences
E. Lansing, MI 48824
(517) 353-3185
(517) 353-8924 FAX

Sadik Tuzun
Auburn University
Auburn, AL 36849
(205)844-1997
(205)844-1947FAX

Anthony Waiss, Jr.
USDA
800 Buchanan Street
Albany, CA 94710
(510) 559-5820
(510) 559-5777 FAX

Phillip J.Wakelyn
National Cotton Council of America
1521 New Hampshire Avenue, NW
Washington, DC 20036
(202) 795-7805
(202) 483-4040 FAX

Ralph Waniska
Texas A&M University
Dept. Of Soil & Crop Sciences
College Station, Tx 77843-2474

Arthur Weissinger
North Carolina State University
Dept. Of Crop Science
P.O. Box 7620
Raleigh, NC 27695-7690
(919)515-2704
(919)515-7959FAX

Don White
University of Illinois
Department of Crop Sciences
1102 South Godwin Avenue
N425 Turner Hall
Urbana, IL 61801
(217) 333-1093
(217) 244-1230 FAX

Don Wicklow
NCAUR/ARS - USDA
1815 N. University Street
Peoria, IL 61614
(309)681-6243
(309)681-6686FAX

Neil W.Widstrom
USDA-ARS-IBPMRL
P. O. Box 748
Tifton, GA 31793
(912) 387-2341
(912) 387-2321 FAX

Paul Williams
USDA-ARS
Box 9555
Mississippi State, MS 39762
(601) 325-2735
(601) 325-8441 FAX

Dave Wilson
Coastal Plains Exp Station
Dept. Plant Pathology
Tifton, GA 31793
(912)386-3368
(912)386-7285FAX

Gary L. Windham
USDA-ARS
Box 9555
Mississippi State, MS 39762
(601) 325-7795
(601) 325-8441 FAX

Charles Woloshulk
Purdue University
1155 Lilly Hall
West Lafayette, IN 47907
(317) 494-3450
(317) 494-0363 FAX